FACTORS CONTROLLING ERYTHROPOIESIS

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Factors Controlling
ERYTHROPOIESIS

By

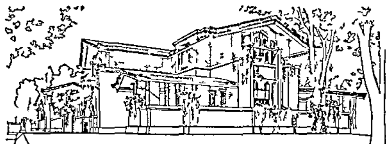
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To our wives

PREFACE

Students of hematology have long recognized the basic need for knowledge of the physiologic and pathophysiologic mechanisms which govern erythropoiesis Although the subject has received the attention of investigators for many years the theories which arose were considered by most to be speculative and lacking in experimental support Research activity has progressively increased during the last decade and studies in many laboratories have now confirmed beyond reasonable doubt the existence and importance of a humoral erythropoietic regulatory mechanism In spite of recent advances the problem of the control of erythropoiesis has not been solved Areas of disagreement and controversy currently characterize this field of hematologic research The confusion which exists is not altogether surprising *in view of the varied experimental approaches that have been* used and the overall complexity of erythropoiesis However it is vital that these apparent discrepancies be explained

The preparation of this monograph was prompted by the wide spread interest in the regulation of erythropoiesis and by the desire to reconcile insofar as possible certain divergent observations This project was undertaken with the realization that many questions remain unanswered and that future studies will probably necessitate revision of some of our current concepts It is impossible on the basis of available data to reach definitive conclusions concerning all phases of this subject Consequently this book represents our evaluation of the present status of the role of humoral factors in the control of erythropoiesis It is not in all instances a clear cut exposition of fact The latter must await further experimental documentation Even so many points have been clarified by recent findings Studies designed to more accurately define the homeostatic mechanisms which govern erythropoiesis and the formation of other hemic elements are now in progress in many laboratories More information should soon be

PREFACE

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The preparation of this monograph was prompted by the widespread interest in the regulation of erythropoiesis and by the desire to reconcile, insofar as possible, certain divergent observations. This project was undertaken with the realization that many questions remain unanswered and that future studies will probably necessitate revision of some of our current concepts. It is impossible, on the basis of available data, to reach definitive conclusions concerning all phases of this subject. Consequently, this book *represents our evaluation of the present status of the role of* humoral factors in the control of erythropoiesis. It is not in all instances a clear cut exposition of fact. The latter must await further experimental documentation. Even so, many points have been clarified by recent findings. Studies designed to more accurately define the homeostatic mechanisms which govern erythropoiesis and the formation of other hemic elements are now in progress in many laboratories. More information should soon be

forthcoming In addition to their fundamental physiologic significance the clinical implications of such research activities are readily apparent

The multiple contributions of the many other workers in this field are gratefully acknowledged We have attempted to include most of the recent additions to the rapidly expanding literature on this topic but the bibliography is not intended to represent an inclusive review We are especially indebted to Doctor Donald R Korst for his help with the radioisotopic studies and to Miss Martha J Long Miss Marilyn C Grayhack and Mrs Helena K Tascott for their excellent technical assistance Our experiments were supported in part by Contract No AT(11 1)–75 from the Atomic Energy Commission and by Research Grant No A 1991 from the National Institutes of Health United States Public Health Service

J W L
F H B

ADDENDUM

Doctor Frank H Bethell died suddenly in Ann Arbor Michigan on April 21 1959 We had actively collaborated for several years in our studies on the humoral control of erythropoiesis and had made plans for the final work on this manuscript the day of his untimely death It was with great personal sorrow that I had to proceed with these plans alone We who were privileged to work closely with Doctor Bethell and to know him intimately shall long cherish his memory

JAMES W LINMAN

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FACTORS CONTROLLING ERYTHROPOIESIS

Chapter I

GENERAL CONSIDERATIONS OF THE RED BLOOD CELL

"The history of the red corpuscles starts at the beginning of the 17th century. About 1610 in the small Dutch town of Middle-bourg an obscure optician named Zacharie Jans made spectacles and polished lenses. It is related that his children while playing in his workshop were directed by chance to pile the lenses like bricks one on top of the other revealing unconsciously to the suddenly inspired father the inherent possibilities of the combination of lenses and the birth of the microscope."[1]

Although the precise details surrounding the invention and development of the microscope are lost, the above account of this monumental discovery emphasizes the far-reaching implications of seemingly unimportant and unrelated observations. Thus the science of hematology as we know it today may have had its beginning in the humble workshop of an astute and observant lens maker. Following the adaptation of the microscope to biologic investigation by Galileo (Pisa 1564-1642), the stage was set for the subsequent identification of the erythrocyte.

The red cells in human blood were discovered by the Dutch microscopist Leeuwenhoek in 1673, although Swammerdam described the oval red cells of the frog in 1658 and Malpighi noted their presence in 1665 but mistook them for fat globules. Approximately 100 years later William Hewson published an erudite description of the form, structure and dimensions of the red corpuscles. However, it was not until the latter half of the 19th century that the true physiologic significance of the erythrocytes became apparent. Hoppe-Seyler's demonstration (1867) that the hemoglobin contained in the red blood cell possessed the property of taking up and releasing oxygen stimulated investigative activity and a number of important advances followed.

Neumann (1868) first recognized that the circulating erythrocytes were formed in the bone marrow from colorless nucleated

precursors Following Ehrlich s contributions and the advent of staining techniques study of the morphologic characteristics of the formed elements of the blood and the blood forming organs was greatly facilitated Concomitant with the development of knowledge concerning the structure and function of the red blood cells procedures were devised to measure the peripheral erythroid values Although originally crude these techniques were constantly modified and improved by many workers thereby permitting accurate quantitative determinations As a result it became possible to study all aspects of erythropoiesis Prior to considering the control of erythropoiesis however it would seem desirable to review briefly the origin development structure components, and function of the red blood cell [7]

Mesenchyme the embryonic connective tissue constitutes the ontogenetic anlage of the mammalian erythrocyte and the first blood cells are formed in the numerous blood islands of the yolk sac These mesoblastic elements differentiate in two directions The peripheral cells form the endothelial lining of the first blood vessels whereas those that are more centrally located become the pluripotential primitive blood cells or hemocytoblasts i e cells producing blood cells In the human embryo, the mesoblastic phase of hemopoiesis continues throughout the first two months of embryonic life and primitive erythroblasts or hemoglobin containing cells predominate Intravascular blood formation is then replaced by the hepatic phase which is characterized by active erythropoiesis in the spleen and thymus as well as the liver and by the appearance of granulocytes and megakaryocytes

During the fifth month of gestation the final or myeloid phase of hemopoiesis begins Proliferation of mesenchymal cells causes resorption of the cartilage of the bone primordia and forms islets or centers of blood formation Initially, the bone marrow is largely concerned with granulopoiesis and erythropoiesis continues to take place chiefly in the liver Splenic erythropoiesis terminates near the end of the fifth month However the bone marrow gradually takes over all aspects of hemopoiesis At birth intramedullary blood formation has entirely supplanted that occurring in extramedullary sites The mesenchymal cells are reduced to a minimal reticular stroma by the growth of myeloid

elements but these polyvalent cells persist throughout life with all of their embryonic potentialities intact Normal blood counts are attained only after birth but the transition from embryonic to postnatal life is not marked by any sudden changes in hemopoiesis The processes of differentiation and proliferation previously established continue without interruption

Controversy in regard to the origin and inter relationship of the blood cells has existed for many years and given rise to the so called monophyletic dualistic and polyphyletic theories of hemopoiesis The problem is still disputable but available knowledge indicates that erythrocytes granulocytes megakaryocytes and probably monocytes are derived from the multipotential myeloid reticulum cells or hemocytoblasts Although normally present in only small numbers these cells may be quite numerous in conditions associated with marrow hyperplasia

Multiplication of myeloid elements after differentiation into a certain cell lineage occurs chiefly if not entirely by homeoplastic mitosis i e one cell gives rise to two similar daughter cells Consequently the majority of the myeloid elements are the products of homeoplasia Nevertheless if one accepts at least a modified monophyletic origin of the blood cells from the primitive pluripotential reticulum cells it must be viewed as basically heteroplastic In addition to the multiplication of specific precursor cells hemopoiesis involves the maturation of these immature elements and the release of mature cells into the circulation The former process is characterized by the evolution of the definitive morphologic and physiologic attributes of each cell type e g the ability to synthesize hemoglobin in the case of cells of the erythrocytic series

Our knowledge of the multiple physiologic activities which culminate in the formation of the mature erythrocyte is incomplete Many experimental models have been devised in an attempt to elucidate the formation of the red blood cells and much information has been obtained from newer cytochemical and radioisotopic techniques However the heterogenicity of the rapidly proliferating myeloid elements makes interpretation of experimental observations quite difficult Even so a number of distinct morphologic and functional stages can be recognized in the develop

ment of the erythrocyte and serve as a useful outline of this process

Erythropoiesis is initiated by differentiation of the pluripotential reticulum cell into an erythrocytic precursor This process must involve a cellular division with one of the resultant cells being diverted into erythropoiesis and the other remaining in an undifferentiated state Otherwise depletion of the multipotential stem cells would occur A phase of rapid cell proliferation and beginning maturation follows The exact number of divisions which the *nucleated erythrocytes undergo is not known but* multiplication shows a progressive increase with differentiation As a result the more mature mitotic cells contribute most of the proliferative activity The growth rate then declines and a stage characterized by a pyknotic nucleus is reached These cells are most likely incapable of further proliferation but cytoplasmic maturation continues

The hemoglobin molecule is actually formed for the most part after mitotic activity ceases The synthesis of hemoglobin is associated with a loss of other cytoplasmic proteins and desoxyribose nucleic acid and mitochondria disappear *The last* vestiges of ribose nucleic acid persist even after the nucleus has been extruded and hemoglobin production continues in the non nucleated reticulocyte However when the ribose nucleic acid has completely disappeared the cell is no longer capable of synthesizing heme or hemoglobin Maturation is then complete

Unfortunately many terms have been employed to describe the various *developmental stages of the erythrocyte based on struc*tural changes and gradually altering morphologic characteristics Because of this varied nomenclature confusion and misunder*standing have arisen For this reason we prefer to use the follow*ing terminology [8]

1) Rubriblast The first recognizable cell of the erythrocytic *series characterized by finely stippled to reticulated nuclear chro*matin and evident nucleoli

2) Prorubricyte A cell of the erythrocytic series in which nucleoli are still discernible but with a *nuclear chromatin structure* too coarse to be classified as a rubriblast

3) Rubricyte A cell of the erythrocytic series possessing de

finitive nuclear chromatin structure but containing no nucleoli This stage may be further subdivided into basophilic polychromatic or normochromatic rubricytes according to the amount of hemoglobin contained in the cytoplasm

4) Metarubricyte A cell of the erythrocytic series which has a pyknotic fragmented or partially extruded nucleus consisting of a dense solid structureless mass

5) Reticulocyte A non nucleated erythrocyte which possesses when supravitally stained (usually with brilliant cresyl blue) one or more granules or a diffuse network of fibrils Without this special staining technique reticulocytes are indistinguishable from erythrocytes

6) Erythrocyte Any non nucleated cell of the erythrocytic series

The end product of erythropoiesis is ideally suited for the efficient completion of the physiologic functions it has to perform Combustion is the essential chemical nature of life and requires a constant supply of oxygen together with the simultaneous removal of carbon dioxide Approximately 250 ml of oxygen are absorbed and 200 ml of carbon dioxide are produced per minute in a human being at rest These quantities are increased many fold during exercise If the respiratory gases were carried in physical solution it has been estimated that man s activity could be only one fiftieth of that which is actually possible Hemoglobin permits the transport of about 100 times as much oxygen as could be carried by the plasma alone In most invertebrates the oxygen carrying pigment exists freely in the plasma where it exerts an osmotic pressure equivalent to five times that of the plasma proteins When hemoglobin is contained within an erythrocyte as is found in the vertebrate phylum the mechanism for the transport of oxygen is greatly improved

Further improvement was achieved during the phylogenetic development of the red cell by the loss of the nucleus and the subsequent reduction in oxygen consumption by the erythrocyte itself In addition the decrease in cell size which occurred in the evolution of the erythrocyte contributed to erythrocytic efficiency Although there is a relatively uniform quantity of hemoglobin per unit volume of blood in all vertebrates the size and num

ber of erythrocytes vary markedly The large cells of the lower vertebrates circulate less freely than the smaller erythrocytes of mammals However the increased surface area of the latter approximately 1500 times that of the body is more important and greatly improves gaseous exchange The biconcave shape of the erythrocyte also aids by permitting the diffusion of gases in and out of the cells with maximal ease Hence the mammalian erythrocyte has attained a high degree of efficiency through the evolutionary process and is particularly well adapted to absorb oxygen rapidly in the lungs pass through the smallest capillaries without damage give up its oxygen readily to the tissues and aid in the removal of carbon dioxide

The mature erythrocyte once thought to be a relatively inert protoplasmic particle consisting largely of water and hemoglobin is now known to be in a continual state of metabolic activity and possesses a finite life span Although the predominant and most important constituent of the red cell is hemoglobin it also contains other proteins lipids vitamins a variety of metals and elements etc In addition a complete glycolytic enzyme system is present and serves to supply the energy requirements of the cell and to maintain hemoglobin in a reduced or functional state Other enzymes and co enzymes include catalase which protects heme from peroxide decomposition and carbonic anhydrase which aids in the transport of carbon dioxide as bicarbonate ions

Hemoglobin comprises about 30 per cent of the wet weight and 95 per cent of the dry weight of an erythrocyte It has a molecular weight of 68 000 and is made up of a protein globin and four prosthetic groups of heme molecules Globin is colorless has a molecular weight of 66 000 and contains all ten of the essential amino acids and many of the nonessential ones[9] The heme moiety is iron protoporphyrin 9 type III a metal complex with an iron atom in the center of a porphyrin structure The protoporphyrin of hemoglobin consists of four pyrrole rings attached to each other by four methene bridges The eight side chains include four methyl two vinyl and two propionic acid groups The methyl side chains are unreactive whereas the propionic acid groups are believed to aid in the attachment of heme to globin and the vinyl groups are apparently essential for the insertion of

iron into the center of the porphyrin nucleus This iron atom is hexacovalent Four of these coordination valences are attached to the pyrrole nitrogen atoms one to the histidine component of the globin molecule and the last attaches itself reversibly to oxygen The four heme moieties of the hemoglobin molecule are colored and impart the red color to hemoglobin The synthesis of globin and protoporphyrin occur independently The former is degraded and returned to the amino acid pool during the catabolism of hemoglobin Iron is also conserved and reutilized in the production of new hemoglobin but the porphyrins are excreted in the bile and must therefore be continuously synthesized

Recent studies[10] have contributed much to our knowledge of the biosynthesis of hemoglobin which is a subject beyond the scope of this monograph The evident complexity of this single aspect of erythropoiesis emphasizes the multiple and intricate physiologic processes involved in the formation of the red blood cell It is readily apparent that the active multiplication and maturation of this highly differentiated series of myeloid cells together with the acquisition of their unique cytoplasmic constituents and enzyme systems requires a carefully regulated chemical system Nucleoproteins globin lipids porphyrins and the numerous other essential components are needed in adequate amounts In spite of these many facets of red cell production a well balanced homeostatic mechanism exists and is capable of maintaining erythrocytic equilibrium and governing erythropoietic responses to a variety of physiologic and pathologic stimuli Individual tissues are thus ensured of a constant and readily available supply of oxygen in amounts needed to support their metabolic activity and sustain life

Chapter II

ENDOCRINE CONTROL OF ERYTHROPOIESIS

The influence of the endocrine glands on hemopoiesis has been the subject of intensive study. Experimental and clinical evidence both direct and indirect, indicating that the hormones are determinants of erythropoietic activity has been recently reviewed by others.[11,1] These data establish beyond reasonable doubt the ability of the secretions of the pituitary, thyroid, adrenals and gonads to affect erythropoiesis. Consequently it is necessary *to examine the possibility that one or more of these hormones* may be of primary importance in the fundamental regulatory mechanism which governs red cell formation. Numerous experiments have been designed ostensibly to test this possibility and it is the consensus that the hormones exert their erythropoietic effects through their modifying influences on metabolic activity. Nevertheless it would seem desirable to consider the evidence favoring this contention before assigning the endocrine glands a secondary role in the control of erythropoiesis.

In order to justify the conclusion that a specific hormone exerts basic control over the erythropoietic process, certain criteria must be met. *A deficiency of such a hormone should be associated with* an anemia and it should be possible to repair or prevent the anemia by appropriate replacement therapy. Conversely an excess should result in accelerated erythropoietic activity and elevated peripheral erythroid values. Furthermore removal of the organ which elaborates the hormone in question should abolish the erythropoietic response to hypoxic stimuli such as lowered barometric pressure or blood loss anemia. All available clinical and experimental observations on red cell production support the thesis that hypoxia or more specifically the relationship between oxygen supply and tissue metabolic requirements constitutes the fundamental stimulus to erythropoiesis (see Chapters III and VII). In experimental animals or human subjects capable of response hypoxia is always followed by increased erythropoiesis

Since cobalt probably imparts its erythropoietic effect by the production of tissue hypoxia (see page 119) the polycythemic response brought about by its administration should also be eliminated by removal of an organ suspected of exerting primary control over red cell production The morphologic and physiologic changes induced in the endocrine glands by hypoxia[10] suggest that they may be involved in the mediation of this erythropoietic stimulus However the demonstration following the application of hypoxic stimuli of increased erythropoiesis in the absence of an endocrine gland would exclude that organ from participating in the erythropoietic response None of the orthodox endocrine glands fulfill all of the above criteria

The thyroid clearly exerts an effect on red cell production as evidenced by the anemia which develops in experimental animals and human subjects after thyroidectomy [14, 17, 19] Moreover anemia is present in the majority of patients with idiopathic myxedema [9] Although not marked the anemia of hypothyroidism is correctable by the administration of dessicated thyroid or thyroxin On the other hand thyroxin is not an effective erythropoietic stimulant in nondeficient recipients Triiodothyronine does enhance the incorporation of iron 59 in normal rats [1] and thyroxin has been reported by some investigators to exert a minimal stimulatory effect on erythropoiesis in normal animals [3, 4] and human subjects [3] In spite of these findings it is not possible to produce a true polycythemia by this means Furthermore patients with hyperthyroidism do not manifest erythrocytosis [6, 8] and thyroidectomized animals exhibit normal erythropoietic responses to lowered barometric pressure[14, 29, 30] and cobalt [31] Therefore it is not possible to assign the thyroid a primary role in the control of erythropoiesis but its modifying influence cannot be denied

Existent data support a similar conclusion in regard to the secondary nature of the erythropoietic effects of the sex hormones A slight to moderate anemia is evident in castrated male animals[14, 3, 33] and eunuchoid men[34] and responds to treatment with testosterone Castration in the female is followed by increased erythropoietic activity preventable by the administration of estrogens [3] Thus testosterone appears to stimulate erythropoiesis whereas estrogens exert an inhibitory effect These observations

apparently explain the slightly higher erythroid values in the males of many species including man. In addition normal male rats recover at a *faster rate from blood loss* than do normal females and regeneration is more rapid in castrated females than in orchidectomized males.[36] Estrogens have also been reported to induce an anemia in normal recipients.[3] Conversely prolonged androgenic therapy in individuals with *carcinoma of the breast* has been associated in a substantial number of patients with the development of considerably greater than normal erythroid values together with myeloid erythrocytic hyperplasia.[34, 39] However orchidectomy does not prevent the erythrocytic response to anemic or hypoxic hypoxia[14, 9, 40] or cobalt[36] and the alterations in erythropoietic activity subsequent to a deficiency or excess of testosterone are minimal. Therefore the gonadal secretions although capable of affecting erythropoiesis and undoubtedly responsible for the normal sex differences in erythroid values are *not the sole or basic determinants of erythropoietic activity*.

The adrenal glands also play a role in the regulation of erythropoiesis. Adrenalectomy produces a slight anemia in experimental animals[13, 14, 19] and corticosteroids induce erythropoietic stimulation in both normal and adrenalectomized recipients.[41, 4, 44, 45] The clinical counterparts of these experimental observations are the anemia in many patients with Addison's disease[1, 49] and the polycythemia in some patients with Cushing's syndrome.[12] However the adrenals are not essential for the maintenance of active erythropoiesis. The maximal depression of hemoglobin levels and *red cell counts reached in the rat about three weeks after removal* of the adrenals is followed by a gradual return to normal values in 60 days.[41] Adrenal cortical extracts prevent the development of this temporary alteration in erythropoiesis thereby establishing their role in its production. The polycythemia produced in recipients of corticosteroids is more impressive than the minimal changes which follow adrenalectomy *but it can be achieved only* by prolonged therapy over a number of weeks.[45] It is not the type of response expected of a primary erythropoietic stimulant. Adrenalectomized animals are also capable of responding *in a normal* fashion to the stimulus imparted by exposure to simulated high altitude,[14, 40, 6] hemorrhage[51] or cobalt.[5] Consequently the

adrenals cannot be considered active participants in the fundamental control of erythropoiesis

There is no doubt that the pituitary gland is intimately related to erythropoiesis and it has been claimed by some to produce a specific erythropoietic hormone distinct from the other hormones elaborated by this organ A moderately severe anemia together with myeloid erythrocytic hypoplasia consistently follows hypophysectomy in experimental animals[11, 13, 15, 19, 9, 30, 53, 5] and is present in patients with panhypopituitarism[1] Since removal of the intermediate or posterior lobes of the pituitary does not produce an anemia[8] the depressed erythropoiesis must be attributed to the absence of the anterior lobe However the bone marrow continues to function under these circumstances and is still capable of responding to a variety of erythropoietic stimuli The erythroid values do not show a progressive decline following ablation of the pituitary Instead they become stable approximately 40 days after surgery and are then maintained at this level for periods as long as a year[55] In addition hypophysectomized rats exhibit active red cell regeneration after hemorrhage[4, 5, 7] or exposure to total body x irradiation[9] with restoration of levels characteristic of the hypophysectomized state The increase in erythropoietic activity is comparable in degree and promptness to that occurring in unoperated animals similarly treated Furthermore it is possible to restore normal values and even produce polycythemia in hypophysectomized rats by the administration of cobalt[9, 60] Even though these animals do not respond to lowered barometric pressures that are capable of evoking erythropoietic stimulation in intact animals[61] e g simulated altitudes of 16 000 feet they are responsive to this hypoxic stimulus[40] At 22 000 feet hypophysectomized rats exhibit positive erythropoietic responses equivalent in magnitude to those observed in normal controls[8] Therefore the pituitary is not obligatory for the typical erythropoietic response to hypoxia but the sensitivity of hypophysectomized animals to this stimulus is decreased

Studies designed to elucidate the pathogenesis of the posthypophysectomy anemia indicate that the pituitary exerts its effects on erythropoiesis chiefly through its various trophic hormones It is obvious that this anemic state is the result of a number of

factors Hypophysectomized animals are capable of responding in some manner to a variety of substances including thyroxin corticosteroids ACTH growth hormone androgens a high protein diet iron and copper[13, 4, 63, 69] Even so it is not possible to restore normal peripheral erythroid and marrow parameters with any of the above forms of treatment given singly Such animals have decreased serum iron levels[70, 71] but storage iron is actually increased and therefore available but not utilized for hemoglobin synthesis Combination therapy with thyroxin and cortisone does repair the anemia in hypophysectomized animals but marrow hypoplasia persists[15, 54, 55, 6, 68, 69] In this regard combined thyroidectomy and adrenalectomy are followed by an anemia similar to that produced by hypophysectomy alone[69, 7] but the marrow is more cellular This difference is apparently due to the continued presence of growth hormone in thyroidectomized and adrenalectomized animals

Growth hormone induces myeloid erythrocytic hyperplasia and reticulocytosis in hypophysectomized rats but it does not eliminate the anemia[47, 4, 5, 66, 6, 73] When this hormone is given to these animals in addition to thyroxin and cortisone all aspects of the posthypophysectomy anemic state are alleviated[5, 68] Thus the erythropoietic alterations secondary to removal of the pituitary apparently result from the absence of adrenocorticotropic thyrotropic and growth stimulating hormones Since none of these hormones appear to be a primary erythropoietic determinant it must be assumed that the pituitary also exerts a secondary effect on erythropoiesis

The evidence that has been responsible for the postulation of a specific pituitary erythropoietic factor deserves comment This concept originated with Flaks and co workers[74] They gave an extract of bovine pituitary orally to hypophysectomized animals and reported that it stimulated erythropoiesis These studies were extended and supported by Contopoulos and his associates[7] Although unable to extract an erythropoietically active principle from the beef hypophysis they were successful in obtaining such an agent from the anterior lobe of sheep pituitary glands In a series of articles[14, 76, 75] the latter group of investigators reported that this substance corrected the anemia secondary to hypoph

sectomy and produced polycythemia in both hypophysectomized and intact recipients In addition they concluded that it did not require the presence of the adrenals thyroid or gonads in order to exert its erythropoietic stimulatory effect However other workers were unable to confirm these observations and difficulties were encountered in preparing a pituitary extract that did not contain corticotropic activity [9,80]

Since ACTH is capable of stimulating erythropoiesis in both normal and hypophysectomized recipients the existence of a discrete pituitary erythropoietic hormone is dependent on its separation from ACTH This separation has not been accomplished and it has been concluded that the terms corticotropin and pituitary erythropoietic factor must be considered synonymous [80] As a result the evidence supporting a direct erythropoietic effect of the pituitary is reduced to the observation that ACTH or pituitary extracts containing this substance can stimulate erythropoiesis in the absence of the adrenals [15,17] Adrenalectomized animals have high levels of endogenous corticotropin activity in the absence of any evidence of erythropoietic stimulation [81] Furthermore it has not been possible to demonstrate repeatedly an erythropoietic effect of corticotropin or anterior pituitary extracts in the absence of the adrenals [80] Unless this can be done it would appear that the concept of a discrete pituitary erythropoietic factor must be abandoned

Observations such as those of Pihero[40] on pleuriglandular deficient animals exclude the possibility that the hormones act conjointly to exert primary control over erythropoiesis Since the endocrine glands apparently do not constitute the chief regulatory center of erythropoiesis or directly affect the myeloid reticulum some other explanation for their erythropoietic activity must be sought There exists strong experimental support that it is directly related to their effects on general metabolism which in turn determine oxygen requirements In this manner increased oxygen need subsequent to an overall increase in metabolic activity would result in a state of relative tissue hypoxia the generally acknowledged fundamental erythrocytogenic stimulus even in the face of normal erythroid values and normal oxygenation of the blood Conversely a reduction in metabolic activity would produce in

the presence of a normal oxygen supply a situation analogous to that created by transfusion polycythemia[82, 83] or hyperoxia[84, 85] both of which depress erythropoiesis

In support of this hypothesis the observed erythropoietic effects of hormonal deficiencies can be correlated with alterations in metabolic activity and oxygen consumption The role of the thyroid gland as a determinant of the rate of metabolism is of course clear cut and hypothyroidism is associated with decreased oxygen consumption Furthermore the appearance of anemia in experimental animals or patients subjected to thyroidectomy coincides with the drop in their metabolic rate and improves as metabolic activity and oxygen utilization are increased following replacement therapy[17] The anabolic effect of testosterone could also explain its influence on erythropoiesis and the curve of the erythrocytic response in eunuchoid men treated with testosterone has been shown to parallel the increase in their BMR[31] The adrenal corticosteroids also contribute to normal heat production[86] Adrenalectomy is accompanied by decreased oxygen consumption and cortisone or hydrocortisone are capable of restoring the metabolic rates of thyroidectomized animals to normal Hypophysectomy is also followed by decreased oxygen consumption which persists in spite of transfusions[5, 87] Therefore it cannot be due to the decreased oxygen carrying capacity of the blood but must be the result of a reduction in metabolic requirements In hypophysectomized animals the metabolic rate and the anemia but not the marrow hypocellularity can be corrected by the simultaneous administration of thyroxin and cortisone

The erythropoietic stimulation caused by hormonal excesses may be related to the increased metabolic activity so induced The relative ineffectiveness of thyroxin as an erythropoietic stimulus in normal animals the absence of polycythemia in patients with hyperthyroidism and the prolonged treatment needed to produce polycythemia with ACTH the corticosteroids or testosterone do not necessarily detract from this theory It is obvious that many factors must influence the final response Certain compensatory measures e g the tachycardia increased cardiac output and hypercapnia which accompany hyperthyroidism may be sufficient to maintain an adequate supply of oxygen without

significant elevation in the erythroid values Thyroxin corticosteroids and testosterone all increase oxygen requirements In the case of the pituitary it would appear to exert its erythropoietic action through its effect on the thyroid and adrenals ACTH does accelerate calorigenesis and erythropoiesis but only in the presence of the adrenals [80, 86]

The single exception to this apparent relationship between the metabolic and erythropoietic activities of the various hormones is the growth stimulating hormone of the anterior pituitary Although capable of producing a reticulocytosis and augmenting myeloid erythrocytic cellularity in hypophysectomized animals this hormone fails to correct the anemia and does not increase oxygen consumption [54] Since growth hormone can influence the marrow without altering oxygen consumption the possibility that it may act directly on the marrow appears likely However this substance cannot be considered a primary erythropoietic stimulant

It is therefore concluded that the endocrine glands exert secondary control over erythropoiesis through their effects on metabolism and oxygen requirements Even though they serve to determine in this manner the level at which erythrocytic equilibrium should be maintained they do not appear to affect the marrow directly Thus the decreased erythroid values which occur in the presence of hormonal deficiencies may be assumed to represent normal levels for the rate of metabolic activity present instead of a true anemic state In other words erythrocytic equilibrium has been re established at a new level which although lower than normal for the species involved is still adequate to supply sufficient amounts of oxygen to fulfill the decreased needs of the tissues This concept is supported by the normal cardiac output in patients with hypothyroidism and anemia [17] whereas acute and chronic blood loss anemia is associated with an enhanced cardiac output [88, 89]

The theory that the endocrines influence erythropoiesis through their metabolic activity will also explain several other observations The latter include the need to expose hypophysectomized rats to lower barometric pressures than are required in the case of normal animals in order to elicit an erythropoietic response and

the increased altitude tolerance of rats previously subjected to thyroidectomy or given antithyroid substances[6] Under these circumstances of reduced metabolism the oxygen requirements would be less acute than in normal animals Consequently a more marked decrease in oxygen tension of inspired air would be required to produce tissue hypoxia or an oxygen supply insufficient to meet the reduced cellular needs

In summary the hormones elaborated by the pituitary adrenals thyroid and gonads (androgens) are clearly capable of exerting regulatory control over erythropoiesis as manifested by the anemia present when they are deficient and correctable by specific replacement therapy In addition these substances also stimulate erythropoiesis in normal recipients but such activity is usually slight and requires in most instances prolonged administration of the hormone before it can be detected However these organs are not needed to mediate the fundamental erythropoietic stimulus of hypoxia For this reason it may be concluded that these hormones do not participate in the primary erythropoietic regulatory mechanism but exert instead a modifying or conditioning influence on erythropoiesis Current experimental data indicate that these secondary erythropoietic hormonal effects are related to the role of the endocrine glands as determinants of general metabolic activity and cellular oxygen requirements

Chapter III

EVIDENCE FOR THE EXISTENCE OF HUMORAL ERYTHROPOIETIC FACTORS

The regulatory mechanisms responsible for the normal erythroid steady state and the nature of the stimuli to accelerated erythropoiesis during periods of increased needs have long attracted the attention of investigators It is evident that the formation maturation and release of erythrocytes from the bone marrow into the circulation are governed by equilibrated forces which maintain normal erythroid values and determine erythropoietic responses to stimuli arising from physiologic or pathologic changes Until recently little was known concerning the mechanisms by which erythrocytic equilibrium was maintained or restored Substances required for the production of erythrocytes such as iron folic acid and vitamin B 12 cannot be considered as exerting direct influences on the rate of erythropoiesis since they do not evoke an increase in erythroid values when administered to normal animals or man Alterations in erythropoietic activity following their administration occur only in the presence of respective deficiencies Endocrine glands the nervous system the spleen hypoxia of erythrocytic tissue itself and humoral factors of unknown origin have all been implicated in the regulation of erythropoiesis

The role of the hormones in the control of erythropoietic activity has been discussed in Chapter II It is evident on the basis of both clinical and experimental observations that the endocrine glands are capable of exerting a modifying influence on erythropoiesis However the hormones cannot be considered as fundamental stimuli to erythropoiesis and their hemopoietic effects are most likely incidental or secondary to their metabolic functions

The evidence for the role of the nervous system in the control of erythropoiesis has been reviewed by Grant and Root[90] Although neurogenic factors like others of endocrine origin appear to modify erythropoiesis perhaps by altering levels of equilibra

tion it has not been convincingly shown that their influence is primary

The spleen which in many respects still fits Galen s description as an organ of mystery is intimately associated with hemopoiesis It is the chief erythrocytogenic organ in lower vertebrates and is an important site of hemopoiesis in human fetal development In man splenic reticulum cells persist throughout life and retain their embryonic hemopoietic potentialities In addition to its phylogenetic and ontogenetic roles in blood formation the spleen also influences medullary hemopoiesis as is shown by the effects of removal of the organ as well as by disturbances affecting it[91-96] Yet many if not all of the hemic changes observed in such situations may depend primarily upon the function or dysfunction of the spleen in the removal of corpuscular elements from the circulation Strong evidence supports the opinion that the spleen by means of humoral factors presumably produced by reticuloendothelial tissue may arrest the development of myeloid cells or inhibit the release of mature elements into the circulation However the search for such an agent or agents has so far proved unrewarding and the existence of a splenic hormone with a physiologic action on myelopoiesis although claimed by some investigators has not been established

The relationship of the oxygen content of arterial blood to erythropoietic activity has been recognized for many years The polycythemic response to high altitude was initially described by Bert in 1878[97] and 1882[98] and confirmed by Viault (1890)[99] and Muntz (1891)[100] Hypoxia as the primary erythropoietic stimulus was first proposed by Miescher in 1893[101] He concluded that a relative degree of bone marrow hypoxia is always present thus maintaining constant erythroid activity From this concept the hypothesis was formulated that the oxygen tension of the blood reaching the hemic organs or its efficiency of transference to erythrocytic tissue determines the rate of erythropoiesis Whereas erythropoietic hyperactivity is known to be evoked by a decrease in the oxygen content of arterial blood regardless of its cause the theory that hypoxia of marrow erythrocytic precursors is itself the stimulus to increased erythropoiesis has been disproved Bone marrow oxygen saturation and tension in acute and chronic hem

orrhagic anemia is normal[10, 103] although accelerated erythropoiesis accompanies these states. Furthermore *in vitro* bone marrow studies have shown that a reduction in oxygen tension depresses rather than stimulates erythropoietic activity.[104, 10] Nor is there any evidence to suggest that increased carbon dioxide tension acts as an erythropoietic stimulus. It is evident, therefore, that the part played by hypoxia in the regulation of erythropoiesis is mediated through some means other than direct action on myeloid elements.

Carnot and Deflandre first described humoral erythropoietic stimulatory activity in 1906.[106, 107] In the course of research on the regeneration of organs, these investigators discovered that the serum of rabbits which had been bled was capable of enhancing erythropoiesis when administered to normal rabbits as evidenced by an increase in the recipients' red blood cell counts. To the hypothetical substance responsible for the stimulatory activity of the serum of bled rabbits they gave the name "hemopoietine." Thus the theory of a humoral erythropoietic regulatory mechanism was born. They further postulated that a dynamic balance existed between "hemopoietine" and another hypothetical substance "hemolysin," with augmentation of the former in states associated with active erythrocyte regeneration. They also suggested that means other than hemorrhage, i.e., exposure to high altitudes, could produce similar effects.

This fascinating concept of a humoral erythropoietic stimulating factor was responsible, in the two decades following the original reports of Carnot and Deflandre, for a number of attempts to repeat their findings. Gerhardt (1910)[108] and Boycott, Douglas, and Price Jones (1911)[109] failed to obtain evidence of such a factor. However, Gibelli (1911)[110] was able to corroborate the findings of Carnot and Deflandre. He also extended the work to other species and first described the presence of an erythropoietic stimulating factor in the sera of animals rendered anemic by the administration of phenylhydrazine. During the same period Muller (1912)[111] and Morawitz (1913)[11] reported equivocal results, although the latter considered that the existence of some chemical agent possessing bone marrow stimulatory activity was highly probable. There followed scattered reports both in accord

and in disagreement with the existence of a humoral erythrocytogenic substance The studies of Forster (1924)[113] were more extensive than those of earlier investigators and he was the first to demonstrate erythropoietic activity in the sera of animals exposed to simulated high altitudes

Little interest was shown in the humoral control of erythropoiesis during the subsequent twenty five years and only sporadic reports of experimental studies appeared in the literature This is understandable in view of the negative findings reported by many workers and the rather marked discrepancies in data which were submitted in support of this hypothesis Although accepted by some the theory of a humoral erythropoietic regulatory mechanism was for the most part considered to be speculative and lacking in experimental confirmation Gordon and Dubin (1934)[114] carried out studies on anemic and anoxic serum and concluded that hemopoietine was not present in such sera Feenders (1936)[11] also obtained negative results but Ter (1938)[116] again demonstrated humoral stimulation of erythropoiesis

After another period of relative investigative inactivity Bonsdorff and Jalavisto (1948)[117] revived the humoral theory Their report led to accelerated interest in this field and was followed by a number of significant studies and observations In a series of experiments involving parabiotic rats Reissmann (1950)[118] was able to show that exposure of one partner to lowered oxygen tension induced accelerated erythropoietic activity in the other animal maintained in a normal atmosphere as evidenced by reticulocytosis increased red cell levels and myeloid erythrocytic hyperplasia This evidence although indirect provided strong support for the existence of a humoral erythropoietic factor Erslev (1953)[119] obtained clear cut evidence of erythropoietic stimulation in rabbits injected with plasma from other rabbits made anemic by repeated bleedings He pointed out the need for giving large amounts of "anemic plasma in order to obtain responses in the recipients This observation undoubtedly explains many of the prior negative results The demonstration in 1954 of erythropoietic activity in boiled "anemic" plasma extracts by Borsook

and associates[1] and by Gordon and his co workers[1] greatly facilitated investigation of the humoral factor With such extracts it has been more feasible to use heterologous species as donors and recipients thereby allowing the testing of plasma from larger animals and man in rats and mice

Since the renewal of interest in this subject research has taken several well defined directions There are currently five approaches to the study of the humoral erythropoietic factors 1) Development of new methods of demonstrating activity which can be performed more quickly more easily and perhaps more quantitatively than those now in use 2) Physical chemical and physiologic characterizations of the substance or substances responsible for this phenomenon directed toward their identification isolation and possible synthesis 3) Detection of the site or sites of production 4) Measurement of activity in pathophysiologic situations such as animals conditioned in various ways and in persons with a variety of disorders involving anemia polycythemia and hypoxia 5) Attempts to quantitate the effects on erythropoiesis of extracts or concentrates of normal plasma which must be done before evaluation of the possible role of decreased production of erythropoietic factors in certain anemias can be undertaken

Although the presence of a humoral erythropoietic regulating mechanism can no longer be denied[1] [1,3] the nature site or sites of production and *modus operandi* in normal and abnormal states relative to erythropoiesis have not yet been clearly defined There are considerable variations in the findings that have been reported by different groups investigating this subject This may be due in part to the existence of two humoral erythropoietic factors which differ in physical chemical and physiologic properties

The two most commonly used names for the humoral erythrocytogenic agent or agents are hemopoietine as first proposed by Carnot and Deflandre[116] and erythropoietin as suggested by Bonsdorff and Jalavisto[117] The latter has received wide acceptance We have been reluctant to employ either term because of the present lack of identification of the substance or substances to which the words refer In addition there is evidence of more than

one factor in biologic materials capable of stimulating erythro poiesis We have therefore preferred to designate these humoral agents as the plasma erythropoietic factors pending the identi fication of their chemical natures physiologic activities and places of origin

Chapter IV

METHODS OF DEMONSTRATING ERYTHROPOIETIC ACTIVITY

The methods of measuring erythropoietic activity in test materials have proved to be one of the major obstacles in the study of the humoral control of erythropoiesis In contrast to *in vitro* chemical tests measurements based on responses of living organisms to substances possessing biologic activity are associated with many variables including species and individual differences It is necessary to know the baseline status of the selected target tissue and to be able to predict with considerable accuracy its responses to active materials thus enabling interpretation of results obtained with substances of unknown activity With adequate controls for each experiment simultaneous testing in a relatively large number of animals and utilization of more than one means of measuring response bioassay techniques can yield valuable information

A single method of demonstrating erythropoietic stimulation possessing all of the characteristics desirable in an ideal experimental situation does not exist These qualities include simplicity speed reproducibility and a yield of specific and quantitative data reflecting the rate of erythropoiesis Since alterations in myeloid erythropoiesis are reflected in the circulating blood changes in the percentage of reticulocytes red blood cell count hemoglobin and hematocrit levels iron 59 erythrocytic uptake and total red cell mass have been widely used as indices of erythropoietic activity In addition quantitative and qualitative examination of the bone marrow affords relevant information However when these parameters are used singly demonstrable changes even though statistically significant do not necessarily constitute a valid or infallible index of true erythropoietic stimulation Consequently the erythropoietic activity contained in test materials can be accurately ascertained only through a detailed analysis of both the peripheral blood and bone marrow of recipient animals

AVAILABLE METHODS

Prior to the introduction of radioisotopic techniques for studying erythrokinetics such as the incorporation of iron 59 in hemoglobin the only means of assessing changes in red blood cell production were measurements of the peripheral erythroid values the reticulocyte responses and marrow erythropoietic activity These determinations together still constitute the best and most reliable means of evaluating all aspects of erythropoiesis The advantages of this method of demonstrating erythropoietic stimulation are 1) The relative simplicity of the laboratory procedures involved 2) The ease with which peripheral blood can be obtained for serial examinations 3) The opportunity to study qualitative as well as quantitative changes in erythropoiesis

Although there are several disadvantages to these techniques most of the criticisms can be met if suitable precautions are taken Two valid objections however are the number of daily injections required and the time consuming nature of the laboratory procedures involved It is apparent that alterations in erythropoietic activity sufficient to produce detectable changes in all of these parameters will not occur immediately Reticulocytosis may be effected after three daily injections but it is necessary to administer an erythropoietically active material for a period of 10 to 14 days in order to elicit significant changes in the peripheral red blood cell levels and bone marrow cytology

Other disadvantages include the inherent errors in the techniques employed the variations of the peripheral erythroid values among normal recipients and the possibility of changes due to factors other than true alterations in erythrocytic equilibrium The statistical and technical errors associated with red cell enumeration are recognized However when erythrocyte counts are performed with strict observance of all details of the procedure[1,4] the likelihood of an erroneous trend is virtually eliminated Individual variations among the recipients can be largely accounted for by information concerning the range of normality for the measurements used and by selection of the recipients with respect to strain sex weight and age The necessity for determining

baseline levels and for having a sufficient number of test animals and controls in each experiment is obvious

The criticism that the changes in the peripheral erythroid determinations or bone marrow findings may not reflect actual stimulation of erythropoietic activity invites scrutiny Examples of such possibilities are the increased erythroid values secondary to a reduction in plasma volume or the release of cells sequestered or stored in sites like the spleen the nonspecific reticulocytosis which may be produced by a number of agents such as potassium arsenite or cholinergic drugs[1,3] and the marrow erythroid hyperplasia and reticulocytosis associated with hemolysis

Hemoconcentration although capable of producing rises in erythrocyte count hematocrit level and hemoglobin measurement does not evoke reticulocytosis or increased numbers of marrow erythrocytic precursors A nonspecific reticulocytosis is not accompanied by elevations in other erythroid values or enhanced marrow erythropoietic activity Myeloid erythrocytic hyperplasia and reticulocytosis caused by hemolysis are not associated with increases in circulating red cell values For these reasons the demonstration of simultaneous increases in erythrocyte counts with or without hemoglobin and hematocrit rises together with reticulocytosis and augmentation in the number of marrow erythrocytic precursors eliminates fluid shifts nonspecific reticulocytogenesis and hemolysis as causes of the observed changes and provides conclusive evidence of erythropoietic stimulation However when any one of these determinations is employed exclusively as the sole method of demonstrating erythropoietic activity in a test material the results must be viewed as inconclusive

The time consuming aspects of these conventional hematologic methods of demonstrating erythrocytogenic activity in biologic materials have been responsible for a search for other means of eliciting similar information The incorporation of iron 59 in hemoglobin and the measurement of total circulating red cell mass by radioisotopic methods have been widely adopted as relatively simple and quick assay methods Although such means of demonstrating erythropoietic stimulatory activity are considerably faster and have yielded valuable information there are certain limitations in their use It is evident that only some of the

quantitative aspects of erythropoiesis can be assessed by such measurements and qualitative changes cannot be detected Augmentation in hemoglobin synthesis is manifested by the radioisotopic techniques but alterations in individual cell size and hemoglobin content are not revealed This fact assumes considerable importance in view of the evidence that there are at least two erythropoietic factors which vary in their chemical physical and physiologic properties (see Chapter V) Furthermore the demonstration of enhanced uptake of iron 59 in the erythrocytes of recipient animals does not constitute conclusive proof that true erythropoietic stimulation has been induced It must be borne in mind that hemolysis results in a similar finding

Hodgson and his associates[1 8] have recently emphasized other hazards that may be associated with the use of iron 59 incorporation alone as an index of erythropoietic activity They have proposed the use of plasma iron turnover determinations which are apparently independent of plasma iron concentration as a more reliable means of detecting the presence of erythropoietically active substances in test materials These workers have reported data indicating that certain plasma extracts may affect iron metabolism and lower cold plasma iron levels in recipient animals without enhancing hemoglobin synthesis Such a mechanism could lead to increased iron 59 incorporation and erroneous conclusions in regard to the erythropoietic activity of the substance being tested

In spite of their limitations and the need for caution in the interpretation of experimental results the convenience of the isotopic tracer techniques are such that these methods constitute valuable research tools in the study of the plasma erythropoietic factors The advantages of the iron 59 techniques include the speed with which an assay can be completed and the few injections and consequently the small amounts of test plasma or serum required

In respect to the species of the recipient or test animal rabbits and rats have been most extensively used but monkeys[1 7] mice[111 1 8 131] guinea pigs[110] dogs[110] and man[106 1 3 13 137] have also been employed Due to the lack of species specific responses selection of the recipient animal appears to be relatively un

important. The necessity of administering a large amount of plasma or serum has in many instances precluded the use of more than a few recipients in any single experiment. This problem has been largely circumvented by the use of heat denatured plasma extracts which have made it possible to employ larger animals or human subjects as donors and smaller animals, usually rats, as recipients.

Recipient animals have been treated prior to testing in various ways in order to demonstrate more readily their responsiveness to erythropoietic stimulation. The earliest experiments were concerned with the rate of restoration of normal erythroid values in animals rendered anemic by bleeding. However, these animals already have accelerated erythropoietic activity secondary to blood loss, and differentiation between the effects of endogenous and exogenous erythropoietic factors in bled recipients is impossible. Furthermore, there is experimental evidence to indicate that there is a point of maximum dose response to the administration of erythropoietic stimulatory materials. The ability to detect augmentation in red cell formation following the administration of additional amounts of the plasma factors to animals that already possess enhanced plasma activity is therefore improbable. Moreover, the production of a change in the erythroid steady state is more indicative of specificity of action than the modification of a previously accelerated rate of red cell formation.

The use of recipients conditioned in such a way as to depress their erythropoietic activity, however, has advantages, particularly in respect to incorporation of labeled iron in new hemoglobin. Although this method has been employed in normal rats,[138, 139] the uptake of iron 59 in the erythrocytes of these animals is so great and variable that it is often difficult to elicit significant differences between the controls and the recipients of potent plasmas. Consequently, it is important to depress erythropoiesis and the incorporation of iron 59 in hemoglobin prior to the injection of the test plasma, thus allowing the development of easily demonstrable differences between observations on control animals and those receiving active materials. Depression of erythropoietic activity can be accomplished by hypophysectomy,[140-144] starva

tion[1,7,14,14,146] transfusion polycythemia[14,147] total body x irradiation[148] or the administration of nitrogen mustard[149,1,1]

Procedures designed to depress erythropoiesis serve no useful purpose when measurements of erythrocytes hemoglobin hematocrit reticulocytes or myeloid erythrocytic precursors are used to test stimulatory activity Although the uptake of iron 59 in hemoglobin is curtailed to 100 per cent of the administered radioactivity the above determinations have an almost unlimited potential for increase For this reason together with the requirement for significance of changes of an equivalent magnitude over the baseline values regardless of their actual level the introduction of variables associated with depression of the recipient's erythropoietic activity is not warranted

Other means of demonstrating erythropoietic activity include a technique recently described by Matoth and co workers[1] These authors studied the effects of sera from patients with anemic and hypoxic hypoxia on the proliferative activity of human erythrocytic precursors *in vitro* In brief their method consists of the culture of human bone marrow suspensions in plasma clots to which small amounts of normal or hypoxic sera are added After incubation in a roller apparatus for 17 hours at 37 C the clot is dissolved by the addition of trypsin solution and absolute nucleated red cell counts are obtained These determinations are then compared with each other and their respective baseline values An increase in erythrocytic elements in the cultures containing hypoxic sera serves as an index of the erythropoietic stimulatory activity of that material These investigators noted about equal numbers of nucleated red cells before and after culture with normal serum and interpreted this observation as evidence that cell proliferation had occurred in these cultures or a drop would have been evident due to maturation to later forms and cell death A significant increase in these cellular elements was observed after culture with a number of sera from patients having hypoxemia secondary to congenital heart disease and with anemias of varying etiology Such an *in vitro* assay offers several advantages including the small amount of plasma or serum needed for study the opportunity to observe quantitative changes in erythropoietic tissue and the testing of human material in a homologous system

Other investigators using different techniques have also reported an increase in the number of erythrocytic precursors in bone marrow cultures containing "anemic" plasma or serum[1 3-14] Although *in vitro* culture techniques hold considerable promise as an investigative tool in the study of the humoral erythropoietic factors and certainly deserve thorough exploration it should be pointed out that many technical problems exist These include among others the difficulties associated with obtaining uniform cell suspensions and accurate counts together with the development of techniques conducive to both proliferation and the maturation of erythrocytic precursors

The enhanced uptake of iron 59 by *in vitro* marrow cell cultures following the addition of active test materials has also been used as a method of demonstrating increased erythropoietic activity[1 -1-8] Even though the initial observations suggested that it did reflect altered rates of hemoglobin synthesis many possible sources of error are evident Jandl and his associates[1 3] were unable to observe any differences in iron uptake by reticulocytes in the presence of "anemic" plasma or "normal" plasma These workers have concluded that the uptake of iron is not always linked to heme synthesis and have shown that iron bound to the iron binding protein is readily and preferentially transferred to immature but not mature erythrocytes whereas free cationic iron is adsorbed onto the membranes of either mature or immature red cells The latter iron but not the former can be eluted with ethylenediaminetetracetic acid Only the immature cells are able to incorporate this membrane-bound iron into heme In addition inorganic iron forms hydroxy compounds which are colloidal and might be spun down as particulate matter and create the false impression that it had been taken up by the marrow cells For these reasons variations in free and protein bound iron available to marrow cells in culture may greatly influence the results Therefore the interpretation of alterations in the uptake of iron 59 by *in vitro* marrow cultures is complex, and conclusions based solely on this finding are subject to question

Gordon and his associates[16 1] have developed a technique for the demonstration of increased erythropoietic activity in the isolat

ed hind limbs of rats These investigators have reported unequivocal increases in the number of nucleated erythrocytic precursors in the marrow of isolated hind limbs of normal rats after perfusion for four hours with the blood from other rats subjected to lowered barometric pressure bled or given phenylhydrazine Perfusion of normal rat limbs with normal blood did not significantly increase the number of erythrocytic precursors as compared to those found in the control or nonperfused limbs The prompt vasoconstrictor response of these isolated limbs to epinepherine the absence of rigor and edema the constant utilization of glucose and oxygen the maintenance of normal biochemistry of the perfusate and the incorporation of C^{14} glycine into radioactive heme isolated from the red cells of the perfusate all indicate that the functional integrity of the limbs is maintained Therefore this method of demonstrating erythropoietic activity would appear to possess many of the advantages of *in vitro* culture techniques without the obvious disadvantages of the latter

According to Rambach Alt and Cooper [16] the incorporation of radioactive phosphorous (P^{32}) into desoxyribose nucleic acid (DNA) of bone marrow and splenic cells reflects accurately the rate of their division and also serves as a useful measure of response of these tissues to erythropoietic factors These workers have determined the relative specific activity of the DNA protein in bone marrow and spleen as well as the distribution of iron 59 in bone marrow red cells spleen and liver following the injection into normal rats of boiled filtrates of plasmas obtained from anemic rabbits

It is readily apparent that a number of varied methods of demonstrating erythropoietic activity are available to investigators studying the plasma factors Each possesses certain advantages and disadvantages and is capable of reflecting changes in one or more of the multiple physiologic processes which encompass erythropoiesis The many techniques that have been devised attest to the overall complexity of red cell formation and emphasize the difficulties associated with attempts to assess alterations in all aspects of myeloid erythropoietic activity

DESCRIPTION OF TECHNIQUES

The majority of our studies have made use of changes in the peripheral erythroid determinations and bone marrow cytology as indices of erythropoietic stimulation Normal Wistar strain rats weighing about 160 grams have been employed most often as recipients The use of younger rats is inadvisable because of their already accelerated rates of erythropoiesis and resultant poor response to additional erythropoietic stimuli[164] Early observations failed to reveal any significant sex differences in response and female rats have been used subsequently since they possess the advantage of a slower growth rate than males The experimental groups have consisted of at least six rats When sufficient quantities of test plasma were available the number has been greater In each experiment a control group of the same size has been given Ringer's solution or normal plasma In some experiments *both Ringer's solution and normal plasma controls have been* employed

In most of our experiments daily injections have been equivalent to 2 per cent of the recipient's body weight with plasma extracts being reconstituted to the original volume of the plasma prior to administration Initially the daily injections were continued for a period of three weeks but more recently the bioassay has been shortened to ten injections given over a two week period (Saturdays and Sundays excepted) All animals are weighed twice weekly and the daily dosage adjusted accordingly Injections are usually given subcutaneously however intravenous or intraperitoneal routes are equally effective

The baseline hematologic studies consist of hemoglobin and hematocrit measurements and erythrocyte and reticulocyte counts Hemoglobins are determined by the cyanmethemoglobin method hematocrits by a microhematocrit technique and erythrocytes are enumerated by hemacytometer Reticulocytes per 1 000 red cells are counted on dried brilliant cresyl blue coverslip films counterstained with Wright's stain The period of observation usually extends for one to two weeks after discontinuation of the injections All determinations are repeated at seven day intervals throughout this time with the exception of the reticulo

cytes which are obtained twice weekly Blood is procured from the tail vein of the rats and an effort is always made to prevent any appreciable loss over that required for the hematologic studies

At the conclusion of the injection period and after the peripheral hematologic values have been determined approximately half of the animals in each group are killed and femoral marrow examined The remainder of the animals can then be followed in regard to the rapidity of the return to normal erythrocytic equilibrium Myeloid erythrocytic hyperplasia represents the most convincing single method of demonstrating changes in erythropoietic activity and a number of techniques for the quantitative study of bone marrow have been devised[16, 109] The following method which we have selected for studying rat marrow has been in use in our laboratories for many years The animals are anesthetized with ether and exsanguinated via the abdominal aorta Both femurs are then removed by cutting through the hip and knee joints with sharp scissors The ends of the femurs are cut off a rubber tube with a mouthpiece is attached to one end and the marrow is expelled onto a watch glass It is occasionally necessary to loosen the marrow with a fine wire This material is then drawn up into a red cell counting pipette diluted with a mixture of half serum (human or animal) and half 4 per cent acetic acid shaken by machine for a period of at least 30 minutes and counted by hemacytometer as for an erythrocyte count One or two drops of serum is added to an approximately equal amount of the remaining marrow and mixed until it is homogeneous and of fluid consistency Coverslip films are made stained with Wright's stain and 500 cell differential counts are done The absolute values for each cell type can then be calculated from the total marrow nucleated cell count This procedure is carried out on the material from both femurs and the counts averaged to obtain the marrow cells per cu mm for each individual rat By use of a serum acetic acid diluent good separation of myeloid elements for counting in the hemacytometer is achieved

As previously emphasized uniformity of the recipients and establishment of the range of normal for the determinations used in measuring changes in erythropoietic activity are prerequi

sites for the interpretation of results The average erythrocyte count hemoglobin level hematocrit value and reticulocyte percentage with their respective standard deviations from the mean of 400 normal female Wistar strain rats weighing 140 160 grams

TABLE I

AVERAGE ERYTHROID VALUES AND MEAN STANDARD DEVIATIONS OF 400 NORMAL FEMALE WISTAR STRAIN RATS

Determination	*Mean*	*Standard Deviation*
Erythrocytes (millions per cu mm)	7 74	0 86
Hemoglobin (grams per 100 ml)	14 7	1 1
Hematocrit (volumes per cent)	44 3	3 0
Reticulocytes (per cent of erythrocytes)	2 9	1 3

TABLE II

MARROW GRANULOCYTIC AND NUCLEATED ERYTHROCYTIC CELL COUNTS MEAN VALUES OF 64 NORMAL FEMALE WISTAR STRAIN RATS

	Total Granulocytic	*Total Erythrocytic*	*Rubriblasts*	*Prorubricytes*	*Rubricytes*	*Metarubricytes*
Cells Per Cu Mm	1 047 150	322 055	3 845	8 960	40 102	241 509
Per Cent of All Nucleated Cells	64 2	17 8	0 2	0 6	2 4	14 5

are listed in Table I The average femoral marrow nucleated cell counts of 64 normal control rats are tabulated in Table II

Before discussing the details of the technique of iron 59 incorporation as a method of demonstrating erythropoietic activity

it would seem desirable to review the various means of condition ing recipients prior to their use as test animals Hypophysec tomy in rats is followed by a great reduction in the uptake of iron 59 in circulating erythrocytes Changes induced by erythro poietic factors may therefore become strikingly apparent in such animals as has been shown by Fried and co workers[140] and by Mirand and Prentice[141] The contention of the former authors that the hypophysectomized rat is actually more sensitive to the effects of anemic plasma factors is not proved since the abso lute rise in the iron uptake in the intact animal may not differ appreciably from that of the hypophysectomized rat Neverthe less it seems plausible as suggested by Fried and his associates[140] that during the first few weeks after hypophysectomy the levels of circulating erythrocytes and hemoglobins are higher than are required for the reduced metabolic status of the animal and that a state of relative plethora is present It may be argued reason ably but without proof that the endogenous production of the erythropoietic factors is thereby reduced and that the responses of the recipient animals to potent materials become more evident although not necessarily exaggerated However Mirand and Prentice[143] used rats 40 days post hypophysectomy at which time hemic equilibrium had been re established They reported results comparable to those of Fried and co workers

Animals are suitable for testing as recipients of erythropoietic factors eight days after hypophysectomy Rats so conditioned are delicate and losses due to intercurrent infections and adverse environmental conditions may be heavy Furthermore they ex hibit erythropoietic responses to a variety of hormones and nu trients (see Chapter II) which might be present in test materials It should also be noted that substances such as ascorbic acid and sodium bicarbonate[30] induce a nonspecific reticulocytosis in hy pophysectomized rats In addition Gordon and his associates[1] have reported increased numbers of erythrocytic precursors in the isolated hind limbs of hypophysectomized rats after perfusion with normal blood In order to obviate these disadvantages as well as to explore different modalities various other methods of preparing animals for iron uptake studies have been devised

Starvation of rats for three days[147] allowing water *ad libitum*

has been found to result in a reduction of iron incorporation in new hemoglobin attributable to lowered oxygen tissue consumption and decreased hemoglobin requirement. Such animals respond to the administration of erythropoietic factors by a greater than five fold increase in iron uptake. However, as the period of starvation is increased from three to seven days, these animals show a greatly diminished response to the injection of active plasmas. This decreased sensitivity is probably due to the overall deterioration of the animals. These animals are also difficult to handle and the possibility exists that they may respond to certain nutrients in the plasma or serum being assayed.

The induction of a polycythemic state by the infusion of red cells in both experimental animals[1,9,14] and man[8] causes an almost complete but temporary cessation of erythropoiesis. The increase in oxygen carrying capacity of the blood over and above that required for normal cellular metabolic activity presumably decreases the endogenous elaboration of the plasma factors and thereby depresses red cell production.[8,1,9,14] Normal rats given approximately eight daily intraperitoneal injections of 2 ml. of washed homologous erythrocytes suspended in saline have hematocrits between 70 and 75 volumes per cent and a definite decrease in the rate of incorporation of iron 59 in hemoglobin.[14] Animals so treated have been shown to be quite satisfactory recipients in the short term assay for the plasma erythropoietic factors. On theoretical grounds, transfusion polycythemia would appear to be the best available means of depressing erythropoiesis in the recipients. Although the other methods are necessarily associated with many variables and must produce effects other than on hemopoietic elements, transfusion polycythemia most likely depresses erythropoiesis through a normal physiologic mechanism. Mice rendered polycythemic by transfusions[1,9] are also suitable recipients.

Rats maintained for 10 days in an atmosphere of 85 to 95 per cent oxygen also display a reduction in the uptake of iron 59 in erythrocytes.[14] The same reason given for the altered myeloid erythropoietic activity secondary to transfusion polycythemia probably explains this finding. Since these animals do not survive in a high oxygen atmosphere for more than two weeks, depres

sion of erythropoiesis by the production of transfusion polycythemia is more practical

Direct injury of myeloid elements is followed by a decrease in red cell production due to fewer numbers of erythrocytic precursors and has also been used to modify the uptake of iron 59 in recipients prior to the administration of erythrocytogenic materials For this purpose x irradiation and nitrogen mustard have been employed as mitotic poisons Stohlman and Brecher[148] have shown that sublethally irradiated rats have decreased iron 59 uptake in erythrocytes Active plasmas administered to these animals induce a clear cut increase in the rate of iron 59 incorporation in hemoglobin

Nitrogen mustard treated rats also have reduced uptake of iron 59 and are suitable recipients for demonstrating erythropoietic stimulatory activity[149,150] A satisfactory dose which produces sufficient depression of erythropoiesis for the purpose intended is 0 4 mg of nitrogen mustard per kilogram of body weight It is important that the iron 59 be given within a 48 hour period following the administration of nitrogen mustard After this interval of time has elapsed the uptake is accelerated presumably due to the regeneration of the marrow The injection of active plasmas evokes in these animals a four to five fold increase in the percentage of radioactivity accounted for in hemoglobin at 18 and 24 hours However as is common to nearly all methods of depressing erythropoiesis in recipient animals prior to the administration of test plasmas or sera there is some variability in the responses of nitrogen mustard treated rats

Although the mechanisms by which red cell production is depressed vary with the method used to prepare the test animals a decrease in the uptake of iron 59 in circulating hemoglobin ensues For the reasons given hypertransfusion would appear to be the most satisfactory means to achieve erythropoietic suppression However with adequate controls any of the other methods will yield valid data The use of conditioned recipients in the radioiron incorporation technique of demonstrating erythropoietic activity is important even though these animals may not show a greater absolute erythropoietic response than do normal animals Such recipients of erythropoietic stimulatory agents do however

have relatively stable and uniform rates of iron uptake as opposed to the wide fluctuations evident among normal rats and in the serial determinations of a single animal The small percentage of injected radioactivity recoverable in the circulating hemoglobin of animals with depressed red cell production allows only slight variation in the individual values Therefore the enhanced iron incorporation induced by active plasmas or sera is more evident and consistent

The methods used by different workers to measure incorporation of radioiron in hemoglobin have been quite uniform Minor modifications involve the amount and number of injections of the test plasma and the intervals of time after the administration of the labeled iron that blood specimens are obtained for counting We have employed the following technique in our studies utilizing this means of demonstrating erythropoietic activity

After the test animals have been prepared by the method selected to depress iron 59 uptake the material to be tested is injected in an amount equivalent to 2 per cent of the recipient s body weight and repeated 6 and 30 hours later Each experiment has included a control group given either Ringer s solution isotonic saline or normal plasma Following the last injection 0 5 ml of ferrous (Fe 59) citrate containing 2 μc of radioactivity is given intravenously into the recipient s tail vein Eighteen 24 and 42 hours later 0 02 ml samples of blood are drawn into capillary pipettes for the measurement of radioactivity and hemoglobin content The specimens so obtained are immediately placed into 5 ml of cyanmethemoglobin or Drabkin s solution and the hemoglobin determined in a photoelectric colorimeter Two 2 ml aliquots of this solution are then placed in scintillation well counter tubes and counted Duplicate standards are prepared by diluting 0 5 ml of ferrous (Fe 59) citrate containing 2 μc of radioactivity to 250 ml in a volumetric flask Three 2 ml aliquots of each are counted in a scintillation well counter and the average taken as the standard for total injected radioactivity Since the iron 59 in 0 5 ml was diluted to 250 ml the total injected counts per minute is the product of the average counts per minute in the 2 ml standards multiplied by 125 The counts per minute per ml of

rat blood are obtained by multiplying the average counts per minute in the 2 ml samples by a similar factor of 125 (0.02 ml of blood is diluted to 5 ml and 2 ml aliquots are counted). Each animal's average hemoglobin is corrected to 15 grams per 100 ml for purposes of standardization. Blood volume is then calculated on the basis of 4.59 ml per 100 grams body weight.[170]

$$\text{Blood Volume (B.V.)} = \text{Weight} \times 0.0459 \times \frac{\text{Hemoglobin}}{15}$$

Utilizing this value and the mean counts per minute (c/m) in 2 ml of the sample and standard respectively, the per cent uptake of iron 59 in the erythrocytes can be calculated from the following formula:

$$\%\ \text{RBC Fe 59 Uptake} = \frac{\text{c/m (2 ml sample)} \times 125 \times \text{B.V.}}{\text{c/m (2 ml standard)} \times 125} \times 100$$

or

$$\frac{\text{c/m (2 ml sample)} \times \text{weight} \times \text{hemoglobin} \times 0.306}{\text{c/m (2 ml standard)}}$$

Measurement of the total circulating red cell volume by the iron 59 labeled cell dilution technique[170] has also been used by a number of investigators to determine the erythrocytic stimulating capacity of test materials. This means of demonstrating erythropoietic activity does not offer any distinct advantage over the iron 59 uptake technique. The results with both of these methods are in agreement and the information so obtained in regard to changes induced in erythropoiesis is comparable. The details of this technique and the other less frequently used methods are readily available in the literature.

SUMMARY AND CONCLUSIONS

Hemoglobin and hematocrit determinations, erythrocyte counts, reticulocyte response and marrow cytologic studies in normal rats given 10 to 14 daily injections of an erythrocytogenic mate-

rial is the method best suited to yield direct evidence of both quantitative and qualitative changes in erythropoietic activity. However, the number of daily injections and the total amount of plasma or serum required to elicit definitive evidence of erythropoietic stimulation by this technique are so great that it is practical only when heterologous plasma extracts from larger donors are given to smaller test animals.

Study of unmodified plasma, whether from a donor of the same or different species than the recipient, usually necessitates the use of a short term assay technique such as the incorporation of iron 59 in hemoglobin. This method permits the study of relatively small amounts of homologous material and, as has been generally assumed, the use of unmodified heterologous plasma without the induction of a foreign protein reaction. A recent study by Lowy and his co-workers[131] casts doubt on the validity of the latter statement. These investigators have reported that boiled filtrates of anemic rabbit plasma produce polycythemia in recipient rats. However, several injections of the unmodified parent plasma induced hemolysis and a resultant decrease in hemoglobin. Similar results were observed following the administration of whole normal rabbit plasma to recipient rats, but the boiled filtrate of these plasmas had no effect. They concluded that this hemolytic action is related to the development of antibodies in the rats given unmodified rabbit plasma and recommended that such materials be heated to destroy the antigenicity of the plasma proteins prior to testing. Plasma brought quickly to 75° C and immediately cooled was found to retain full erythropoietic activity without sensitizing recipients of a different species.

In view of the above observations, we agree that heterologous plasmas should be heated prior to testing. Short term assay techniques which utilize iron 59 incorporation or reticulocyte response as a measure of erythropoietic stimulation would be altered by hemolysis, thereby obscuring the true significance of the results obtained. It is important, however, to boil for less than five minutes when using the iron 59 uptake technique. Our studies and those of others indicate that prolonged boiling will inactivate the agent in active plasma which stimulates hemoglobin synthesis (see page 75).

Although iron 59 uptake studies are capable of demonstrating augmentation in hemoglobin synthesis they do not serve as an infallible index of true erythropoietic stimulation and fail to reflect changes in other aspects of erythropoiesis such as those which determine cell size and hemoglobin content Therefore this assay method is most useful as a rapid screening procedure for the presence of enhanced erythropoietic activity in various test materials or in situations where insufficient plasma precludes the longer and more tedious technique utilizing changes in the peripheral erythroid values and marrow cytology as indices of erythropoietic stimulation However as a preliminary indicator we believe a reticulocyte count in rats or mice given three to four daily injections of active materials is just as informative and considerably less complex

Alterations in recipient animals of individual parameters even though capable of reflecting increased erythropoiesis cannot be construed as unequivocal evidence that the agent being studied possesses true erythropoietic stimulatory properties Thus an increase in iron 59 uptake or plasma iron turnover may be due to hemolysis or the former may result from a lowering of the cold plasma iron level by the test substance reticulocytosis might reflect hemolysis or a nonspecific response myeloid erythrocytic hyperplasia could be caused by hemolysis and augmentation in either hemoglobin or hematocrit levels red cell counts or total circulating red cell volume can occur with hemoconcentration or release of sequestered or stored erythrocytes

On the other hand the failure of test materials to enhance iron 59 incorporation in hemoglobin or to augment the hemoglobin hematocrit or total red cell mass does not necessarily mean that the substance is devoid of erythropoietic activity These measurements may remain stable in the face of an increased rate of production of small cells (see Chapter V) For these reasons caution must be employed in the interpretation of experimental results based on increases in single determinations such as iron 59 uptake reticulocyte percentage hemoglobin levels etc Although such changes may be the result of true erythropoietic stimulation the possibility that they may be due to other causes cannot be entirely excluded The evidence so elicited must there

fore be considered inconclusive Many of the published obser vations on the humoral erythropoietic factors have used single parameters most notably iron 59 uptake with or without con comitant reticulocyte counts to demonstrate the stimulatory ef fect of certain materials Valuable information has been obtained in this manner but the conclusions derived therefrom although not necessarily inaccurate or invalid should receive additional confirmation

It is apparent that there is as yet no entirely satisfactory short cut available to investigators studying the humoral erythropoietic factors Although the detailed analysis of both the peripheral blood and bone marrow responses in recipients of test materials permits a rather complete quantitative and qualitative assessment of changes in erythropoietic activity it remains a lengthy labo rious method However pending the development of shorter and more informative techniques this disadvantage must be accepted if reliable and significant data are to be obtained

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to the coagulum and it was thoroughly stirred boiled for an additional 5 minutes and refiltered This step was repeated two more times

The initial filtrate and washings were then concentrated in vacuo to the original volume of the plasma Subsequent experiments have shown that this latter step can be accomplished more easily by additional boiling without altering the degree or type of erythropoietic response observed in the recipient In more recent studies therefore we have used this method of concentrating the filtrate obtained from the preliminary boiling and subsequent washings

Since the material at this stage of its preparation was quite turbid in appearance presumably due to the presence of retained protein material precipitation with perchloric acid was carried out The boiled extract was chilled to 5 C and cold 5 5 N perchloric acid was added to a final concentration of 0 5 N This material was then filtered the temperature being maintained at 5 C and the pH was readjusted to 6 0 by the addition of 5 N potassium hydroxide After the mixture had been allowed to stand in the cold for several hours the potassium perchlorate was removed by filtration Following this step the plasma extract was clear in appearance

It has been shown[111] that precipitation with perchloric acid in no way detracts from the erythropoietic activity present in boiled "anemic" plasma extracts However it does prevent the development of areas of induration that are occasionally seen at the site of the injections in rats given boiled plasma extracts not treated in this manner Therefore we have usually employed perchloric acid precipitation in the preparation of plasma extracts for testing Unless specifically stated to the contrary all future references to our experiences with protein free or heat-denatured plasma extracts (often designated PI PE) refer to material prepared in a manner identical to that just described

Thirty six normal female Wistar strain rats weighing 180 to 200 grams were given daily subcutaneous injections of anemic" (by phenylhydrazine) rabbit plasma extract in doses equivalent to 2 per cent of their body weight Eighteen such injections were given over a three week period (Sundays excepted) Another group of 36 similar rats were injected with comparable amounts of Ringer s solution and served as controls The hematologic measurements were obtained by the techniques described in Chapter IV

The results of this experiment are shown in Fig 1 The animals injected with the anemic plasma extract developed erythrocytosis and reticulocytosis but failed to show any associated increase in their hemoglobins or hematocrits All values in the animals given Ringer s solution remained constant throughout the period of observation The reticulocytosis was evident three days after the injections were started and the erythrocytosis in seven days These changes in the peripheral blood were accompanied by a fall in the mean corpuscular volume and the mean corpuscular hemoglobin whereas the mean corpuscular hemoglobin concentration remained unchanged (Table III) The small

Chapter V

CHEMICAL, PHYSICAL, AND PHYSIOLOGIC CHARACTERISTICS OF ERYTHROPOIETIC FACTORS

An agent exists in heat denatured anemic plasma extracts which is capable of altering the erythrocytic equilibrium of normal recipient animals. The preparation of these extracts and an example of the response they induce in normal rats will be described in detail since these data form the foundation for many of our subsequent observations on the plasma erythropoietic factors. Adult rabbits constitute a readily available source of plasma for study and can be rendered anemic by a variety of methods. The two most commonly used techniques are repeated bleedings and the production of an acute hemolytic anemia by phenylhydrazine. The latter method is generally preferred because of the greater technical difficulties and relatively high mortality rate associated with cardiac punctures.

ERYTHROPOIETIC RESPONSE TO "ANEMIC" PLASMA EXTRACTS

The following experiment illustrates the erythropoietic response in normal rats to boiled plasma extracts from rabbits made anemic by phenylhydrazine.

Adult New Zealand rabbits were given daily subcutaneous injections of 1 ml. of a 2.5 per cent solution of phenylhydrazine. After approximately seven such injections, the animals had hemoglobins of 4 to 6 Gm. per cent. They were then anesthetized with ether and rapidly exsanguinated through the abdominal aorta. One part of a 3.8 per cent solution of sodium citrate to 9 parts of whole blood was used as the anticoagulant. The whole blood was centrifuged immediately and the plasma removed and frozen until the extraction process was carried out.

The heat-denatured plasma extract was prepared according to a modification of the method described by Borsook and his co-workers.[1,6] The frozen plasma was thawed and the pH adjusted to 5.5 by the addition of 1 N hydrochloric acid. It was then boiled for 15 minutes and filtered. A volume of distilled water equal to the original volume of the plasma was added

TABLE III

AVERAGE RED CELL MEASUREMENTS OF 36 RATS INJECTED WITH AN "ANEMIC" PLASMA EXTRACT AND A COMPARABLE NUMBER GIVEN RINGER'S SOLUTION

Groups		Baseline	1 Week	2 Weeks	3 Weeks*	4 Weeks	5 Weeks
"Anemic" Plasma Extract	MCV	52.8	41.7	38.0	38.2	50.7	53.7
	MCH	16.9	13.2	12.1	12.3	16.0	17.2
	MCHC	32.1	31.7	31.8	32.1	31.5	32.1
Ringer's Solution	MCV	54.1	55.2	54.6	55.2	52.4	52.8
	MCH	17.4	17.4	17.4	18.1	17.2	17.2
	MCHC	31.9	31.4	32.0	32.8	30.7	32.6

*Injections discontinued
MCV —Mean corpuscular volume (cubic microns)
MCH —Mean corpuscular hemoglobin (micromicrograms)
MCHC— Mean corpuscular hemoglobin concentration (per cent)

At the conclusion of the injection period 12 animals in each group were killed and femoral marrow examined by the technique previously described (see page 34). The increase in total marrow cellularity in the animals with the peripheral erythrocytosis was due solely to erythroid hyperplasia and involved all recognizable precursors (Figs 3 4 5 6 and Table IV). This three to four fold increase in marrow erythrocytic elements illustrates one of the most convincing methods of demonstrating increased erythropoietic activity.

After the injections were stopped the erythrocyte and reticulocyte counts and Price Jones curves of the 24 rats remaining in the experimental group rapidly returned to baseline levels (Fig 1). All animals remained healthy throughout the experimental period, gained weight normally and showed no adverse effects from the injections.

The rapid restoration of normal erythrocyte counts after the injections were stopped coincided with the disappearance of the

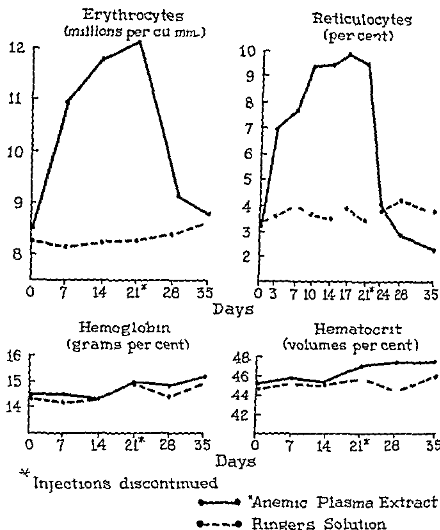


Figure 1 Erythrocytosis and reticulocytosis without significant change in the hemoglobin or hematocrit values of rats injected with boiled extracts of phenylhydrazine induced anemic rabbit plasma *Average determinations* of thirty six animals in each group given the above described materials

size of the erythrocytes was evident on the stained films (*Fig* 2) and in the hemacytometer chamber and could be demonstrated graphically by Price Jones curves constructed from measurements made with a micrometer disc and Wright s stained coverslip films

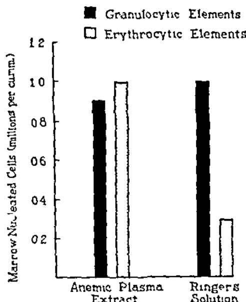


FIGURE 3 Myeloid erythrocytic hyperplasia at the end of a three week injection period in recipients of a boiled extract of anemic rabbit plasma Marrow granulocytic cell counts did not show significant change Average counts of twelve rats receiving the "anemic" plasma extract and a similar number injected with Ringer s solution

solutions These percentages were calculated from the reading for complete hemolysis obtained in distilled water

The second technique used to determine osmotic resistance consisted of the direct enumeration of the erythrocytes remaining intact in media of varying hypotonicity Red cell pipettes were filled as for standard erythrocyte counts using a 1 200 dilution with 0 85 0 65 0 55 and 0 45 per cent sodium chloride solutions respectively as diluents The pipettes were allowed to stand at room temperature for 2 hours shaken by machine at a uniform rate for 3 minutes and the cells were counted by hemacytometer It was then possible to calculate the per cent hemolysis that had occurred in each solution on the basis of the actual number of cells lysed Although a greater number of hypotonic salt solutions would have yielded more detailed fragility curves the possible import of these data did not appear sufficient to warrant the additional blood loss which could in itself have altered erythropoietic activity The counts in 0 85 per cent salt solution were used as baselines to determine the per cent hemolysis occurring in the hypotonic diluents in order to obviate any possible change due to more rapid settling of the cells in the counting chamber These values however did not differ significantly from those prepared with standard red cell diluting fluid

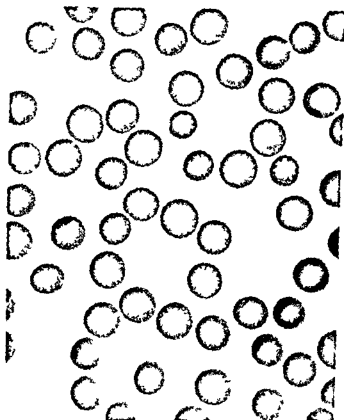


FIGURE 2 Anisocytosis due to the presence of microcytes in a rat injected daily for two weeks with the thermostable fraction of anemic rabbit plasma original magnification x 900

microcytes These observations suggested that the erythropoietic factors contained in boiled anemic plasma extracts induced in nonanemic recipient rats the formation of small cells possessing impaired viability Studies on the erythrocyte osmotic fragility patterns of stimulated rats[17] provide additional support for this hypothesis

Six female Wistar strain rats were given ten injections over a period of 14 days of phenylhydrazine induced anemic rabbit plasma extract A similar number of animals received Ringer's solution and served as the controls In addition to the routine hematologic studies erythrocyte osmotic fragilities were measured by two different quantitative techniques One was the photocolorimetric method of Bethell[1,4] with determination of the per cent hemolysis occurring in 0 65 0 55 and 0 45 per cent sodium chloride

TABLE IV

MARROW GRANULOCYTIC AND NUCLEATED ERYTHROCYTIC ELEMENTS EXPRESSED AS PER CENT OF ALL NUCLEATED CELLS AVERAGE DETERMINATIONS OF 12 RATS IN EACH GROUP INJECTED FOR 3 WEEKS WITH AN ANEMIC PLASMA EXTRACT OR RINGER'S SOLUTION

Groups	*Total Granulocytic*	*Erythrocytic*	*Rubriblasts*	*Prorubricytes*	*Rubricytes*	*Metarubricytes*
Anemic Plasma Extract	41 2	46 2	1 1	2 5	9 6	33 0
Ringer's Solution	62 5	17 8	0 1	0 4	2 1	15 2

turned to normal as had the erythrocyte counts and Price Jones curves (Figs 7 and 8) These findings indicate that only the microcytes possessed this abnormal osmotic behavior

The more commonly used means to measure erythrocyte osmotic fragility are based on the assumption that there is a direct relationship in a given quantity of blood between the number of cells hemolysed and the color imparted to the supernatant The erythrocytosis induced in recipients of erythropoietically active boiled plasma extracts is not accompanied by augmented hemoglobin synthesis Hence such an assumption is invalid under these experimental conditions It is evident that a greater number of these small cells containing less hemoglobin could be lysed without significantly increasing the optical density of the supernatant The determination of erythrocyte osmotic fragility by direct cell counting was first described by Simmel in 1923 [173] The method has received only limited use because of its time consuming aspects together with the fact that it offers no definite advantage in the clinical evaluation of hemolytic states Yet as an investigative tool it is ideally suited to an experimental situation such as the one described above

Disparity in erythrocyte osmotic fragility measurements between the two techniques employed would be anticipated if nor

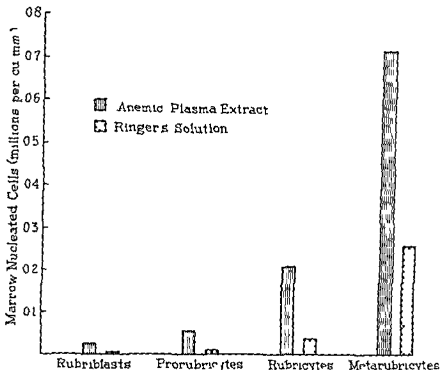


FIGURE 1 Absolute marrow nucleated red cell counts in normal rats injected for three weeks with an anemic rabbit plasma extract The four fold increase in erythrocytic precursors (Fig 3) involved all recognizable stages of erythrocytic development Average counts of twelve animals receiving each of the above described materials

The rats injected with anemic (by phenylhydrazine) plasma extract developed erythrocytosis and reticulocytosis without significant change in their hemoglobins or hematocrits (Fig 7) The microcytes were readily apparent and demonstrable graphically by Price Jones measurements (Fig 8) Erythrocyte osmotic fragility studies with the photoelectric colorimeter failed to reveal a significant variation between the stimulated and the control animals (Fig 9) However osmotic fragility determinations by the direct cell counting technique demonstrated a definite decrease in resistance to lysis in hypotonic media of the red cells in the rats with the erythrocytosis (Fig 10 and Table V) Two weeks after the injections were stopped the osmotic fragility curves had re

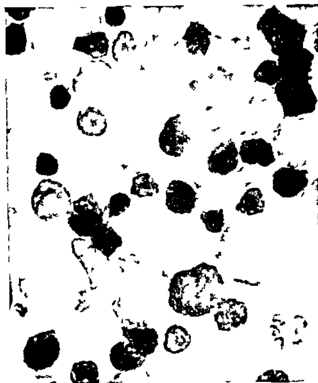


FIGURE 6 Myeloid erythrocytic hyperplasia in a rat injected daily for two weeks with a boiled anemic plasma extract The femoral marrow hyper cellularity in recipients of such test materials is due solely to increased numbers of erythrocytic precursors as reflected by the erythrocyte granulo cyte ratio of 1 1 and a three to four fold increase in the absolute marrow nucleated red cell count original magnification x 900

placed by microcytes in the above experiment they would have contained 25 per cent of the hemoglobin Consequently fragility abnormalities should have been easily detectable by the photo colorimetric technique In addition a larger number of small cells should have been demonstrable by Price Jones measurements The possibility that all of the microcytes in this study did not pos sess decreased osmotic resistance must be considered but the Price Jones curves in the stimulated animals afford evidence

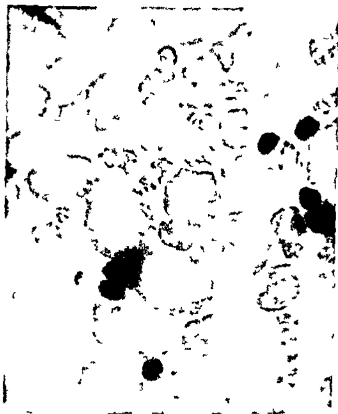


FIGURE 5 Femoral marrow specimen procured from a normal rat showing a ratio of nucleated erythrocytic to granulocytic elements of approximately 1 3 Absolute counts of bone marrow cells in normal rats by the method employed yield approximately 1 000 000 granulocytic and 300 000 nucleated erythrocytic elements per cu mm of femoral marrow original magnification x 900

mal erythropoiesis in the stimulated animals was largely replaced by the formation of microcytes with decreased osmotic resistance It cannot be assumed however that erythropoiesis in normal rats given anemic plasma extracts consists entirely of the production of microcytes Based on a normal life span of 58 to 60 days for rat erythrocytes [1,4,1] approximately 25 per cent of the cells present prior to the administration of the plasma factor would have been removed by natural destructive processes at the end of a 14 day injection period If all of these cells had been re

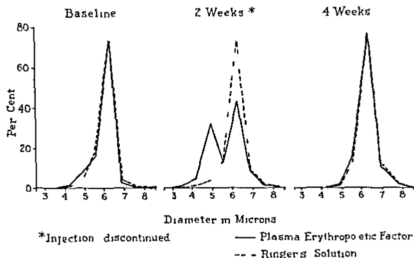


FIGURE 8 Price Jones curves demonstrating graphically the presence of the microcytes responsible for the erythrocytosis (Fig 7) in rats given an anemic" plasma extract The small cells had disappeared two weeks after the injections were discontinued Composite measurements of six rats in each group (From Linman J W and Long M J Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor *Blood* 13 226 238 1958 Reprinted by permission)

against such an occurrence (Fig 8) At the end of the injection period this curve had two definite peaks One represented cells of normal size and the other microcytes The latter comprised 30 6 per cent of the mean erythrocyte count or 3 080 000 red cells per cu mm If all of these cells had been lysed in 0 65 per cent sodium chloride solution the expected per cent hemolysis and erythrocytes per cu mm with this hypotonic solution as diluent would have been 30 6 and 6 980 000 respectively The actual values (Fig 10 and Table V) were 29 1 per cent and 7 030 000 per cu mm

The sources of error inherent in the technique of red cell counting are important considerations in the interpretation of experimental data based on this procedure and have already been discussed It should be re emphasized that with proper precautions the role of such errors can be rendered negligible Furthermore

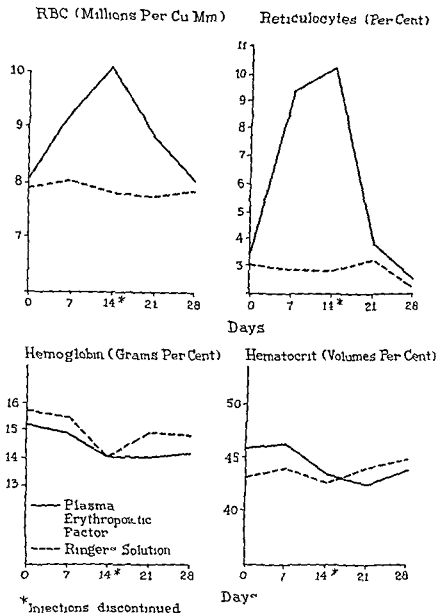


FIGURE 7 Average erythrocyte and reticulocyte counts of six rats injected with a boiled "anemic" plasma extract and six given Ringer's solution The erythrocytosis and reticulocytosis in the former group were not associated with evidence of augmented hemoglobin synthesis (From Linman J W and Long M J Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor *Blood* 13 226 238 1958 Reprinted by permission)

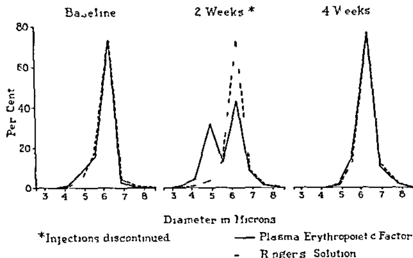


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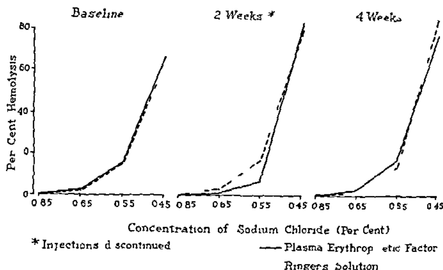


FIGURE 9 Erythrocyte osmotic fragility curves determined by a quantitative photocolorimetric technique There was no significant difference between the measurements in the recipients of the anemic plasma extract and the animals given Ringers solution *Average values of six rats in each group* (From Linman J W and Long M J Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor *Blood* 13 226 238 1958 Reprinted by permission)

the magnitude of the changes in the study just described the similar findings in each rat in the experimental group (Table V) and the constancy of both the individual and average counts in the control group contribute to the significance of these observations There are two other possible sources of error in the direct cell enumeration technique of determining osmotic fragility The first is that lysis may not be an all or none phenomenon and the second is that erythrocyte fragments or ghosts could have altered the counts There is evidence that the former does not occur but if either had been operating in this experiment it would have served to diminish rather than accentuate the findings

Therefore it may be concluded that there exists in the normal rat given boiled anemic plasma extracts a double red cell population consisting of normal cells and microcytes with decreased osmotic resistance The assumption that such an *in vitro* osmotic abnormality indicates an *in vivo* decrease in viability appears jus

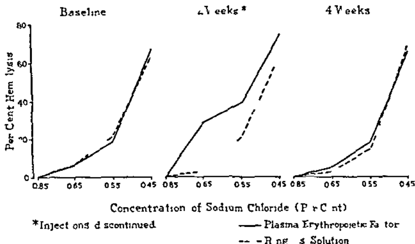


FIGURE 10 Decreased erythrocytic resistance to lysis in hypotonic media in recipients of "anemic" plasma extracts demonstrable by a direct cell enumeration method for determining osmotic fragility Two weeks after the injections were stopped the osmotic fragility curves had returned to base line and control values as had the erythrocyte counts (Fig 7) and Price Jones curves (Fig 8) Average measurements of six rats in each group performed simultaneously with the photocolorimetric technique which failed to show significant variations between the experimental and control animals (Fig 9) (From Linman J W and Long M J Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor *Blood* 13 226 238 1958 Reprinted by permission)

tified Although not strictly analagous to the demonstration of decreased cell survival by radioisotopic techniques the correlation between decreased osmotic resistance and a shortened life span is well accepted in clinical and experimental medicine Decreased cell survival can exist without demonstrable changes in osmotic fragility but the reverse probably does not occur in the intact experimental animal or man A number of variables are involved in the osmotic behavior of red cells and the abnormality responsible for the decreased osmotic resistance of the erythrocytes in recipients of boiled "anemic plasma extracts is not yet apparent The relationship between the basic configuration of an erythrocyte and its susceptibility to lysis in hypotonic media is

TABLE V

STANDARD DEVIATIONS OF THE ERYTHROCYTE COUNTS (MILLIONS PER CU MM) IN DILUENTS OF VARYING HYPOTONICITY MEAN VALUES OF 6 RATS IN EACH GROUP (From Linman J W and Long M J *Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor* *Blood* 13 226 238 1958 Reprinted by permission)

Diluent	*Materials Injected*	*Baseline*		*2 Weeks**		*4 Weeks*	
		Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*
Bethell's Solution	Anemic plasma extract	8.08	0.46	10.06	0.22	8.13	0.15
	Ringer's Solution	7.93	0.44	7.82	0.41	7.82	0.16
0.85% NaCl	Anemic plasma extract	7.94	0.47	9.96	0.33	7.92	0.47
	Ringer's Solution	7.77	0.48	7.85	0.37	7.78	0.16
0.65% NaCl	Anemic plasma extract	7.52	0.42	7.03	0.59	7.59	0.58
	Ringer's Solution	7.24	0.67	7.58	0.31	7.57	0.13
0.55% NaCl	Anemic plasma extract	6.37	0.60	5.94	0.53	6.43	0.45
	Ringer's Solution	5.87	0.80	6.20	0.13	6.57	0.16
0.45% NaCl	Anemic plasma extract	2.52	1.15	2.54	0.59	2.66	0.88
	Ringer's Solution	2.77	0.74	2.71	0.63	2.42	0.52

*Injections discontinued

well established but osmotic fragility is not determined by shape alone The microcytes present in the rats injected with anemic plasma extracts appear spheroidal on dried stained films although

TABLE VI

MEAN RED CELL MEASUREMENTS OF 6 RATS IN EACH GROUP INJECTED DAILY WITH AN "ANEMIC PLASMA EXTRACT OR RINGER S SOLUTION (From Linman J W and Long M J Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor *Blood* 13 226 238 1958 Reprinted by permission)

Measurement	*Anemic Plasma Extract*			*Ringer s Solution*		
	Baseline	*2 Weeks**	*4 Weeks*	*Baseline*	*2 Weeks**	*4 Weeks*
MCV	56 7	43 2	53 7	54 7	54 8	57 2
MCH	18 6	13 9	17 4	19 8	17 9	18 9
MCHC	32 9	32 1	32 4	36 1	32 6	33 0
MCD	6 0	5 7	6 1	6 0	6 1	6 2
MCT	2 0	1 7	1 8	2 0	1 9	1 9
MCS	94 2	80 8	93 4	95 1	94 8	97 1

*Injections discontinued
MCV —Mean corpuscular volume (cubic microns)
MCH —Mean corpuscular hemoglobin (micromicrograms)
MCHC—Mean corpuscular hemoglobin concentration (per cent)
MCD —Mean corpuscular diameter (microns)
MCT —Mean corpuscular thickness $= \frac{4\ MCV}{\pi MCD^2}$ (microns)
MCS —Mean corpuscular surface $= \pi MCD \times MCT + \frac{\pi MCD}{2}$
(square microns)

average cell measurements have failed to reveal a decrease in the length/thickness ratio However these cell indices (Table VI) are mean values and in many instances derived measurements of a distinctly bimodal cell population and do not represent the true size and shape of the microcytes Consequently the behavior of these small cells in hypotonic media might be due to their altered size or shape but intracellular abnormalities injury of the surface membrane a possible combination of the above or perhaps some more obscure factor cannot be excluded

In view of the increased number of reticulocytes present in the stimulated rats the relationship between erythrocyte age and osmotic resistance should be considered but would not seem to be a contributing factor Recent studies indicate that young cells are actually *more resistant to lysis in hypotonic solutions than are* older cells [176 177] Furthermore the number of cells from the rats receiving the anemic plasma extract that were lysed in 0 65 per cent salt solution was three times greater than the absolute reticulocyte count

The rapid re establishment of normal erythrocyte counts after the injections of boiled anemic plasma extracts are discontinued would on the basis of the osmotic fragility abnormalities demonstrable in these animals certainly appear to be the result of the rapid removal of the short lived microcytes and their replacement with a smaller number of normal cells The return of the fragility and Price Jones curves to normal two weeks after the injections were stopped (Figs 8 and 10) strongly supports this conclusion

The failure to demonstrate any significant change in the hemoglobins or hematocrits of recipient animals during the period of *erythropoietic readjustment deserves comment Following cessa*tion of the erythropoietic stimulus afforded by the anemic plasma extract rapid removal of the circulating microcytes might be expected to produce a measurable reduction in the hemoglobin or hematocrit levels The erythroid values were not checked for seven days after the injections were discontinued in order to keep blood loss uniform throughout the period of observation For this reason changes in the immediate post injection period cannot be excluded As previously pointed out however it cannot be assumed that all normal erythrocyte production ceases under these experimental conditions and the microcytes probably contribute relatively *little to the total circulating hemoglobin* In addition these cells most likely have a survival time of approximately two weeks (see page 88) Replacement of the microcytes with normal cells could prevent any discernible drop in the hemoglobins or hematocrits in the early post injection period but it would then be necessary to postulate some augmentation in hemoglobin production during this time which is greater than that

present normally Otherwise the hemoglobin and circulating red cell mass would not be maintained Marrow examinations of stimulated animals show a three to four fold increment in erythrocytic elements involving all recognizable precursors It is not unreasonable to conjecture that following the removal of the erythropoietic stimulus contained in these test materials significant numbers of erythrocytic precursors might mature in an essentially normal manner However augmentation in the endogenous plasma factor activity subsequent to a slight reduction in the oxygen carrying capacity of the blood brought about by removal of the short lived microcytes would seem a more likely explanation for the maintenance of normal circulating hemoglobin levels (see Chapter VII)

Normal rats given multiple daily injections of boiled extracts of erythropoietically active plasmas have consistently manifested erythrocytosis and reticulocytosis without evidence of enhanced hemoglobin synthesis as reflected by the striking stability of their hemoglobin and hematocrit levels even in the face of dramatic increases in the erythrocyte counts Myeloid erythrocytic hyperplasia is also present in the recipients of these plasma extracts and provides together with the erythrocytosis and reticulocytosis conclusive evidence of erythropoietic stimulation The microcytes responsible for the erythrocytosis are easily demonstrable and manifest decreased resistance to lysis in hypotonic media The impaired viability of the microcytes produced in the nonanemic rat in response to this particular stimulus is the apparent explanation for the rapid restoration of normal erythrocytic equilibrium after the plasma extract injections are stopped

THE NATURE OF THE THERMOSTABLE PLASMA FACTOR

The precise nature of the erythropoietic factor in boiled anemic" plasma extracts which induces erythromicrocytosis in normal rats is not known but some of its chemical and physical attributes have been ascertained The presence of erythrocytogenic activity in plasma extracts prepared according to the method described permits in itself several conclusions The substance responsible for this effect is clearly stable over a wide range of temperature and retains its activity after both prolonged boiling and storage

in the frozen state No diminution in the erythropoietic stimulatory effects of extracts prepared from plasmas that have been frozen for periods as long as twelve months occurs Exposure to oxygen does not reduce the activity This factor is acid soluble and not precipitable by perchloric acid Ultraviolet absorption studies have shown that boiling for 30 minutes followed by precipitation with perchloric acid removed 92 3 and 94 1 per cent of the materials absorbed at wave lengths of 260 and 280 millimicrons respectively without altering the type or magnitude of the erythropoietic response in recipients

The thermostable factor is not identical with cobalt which produces a true polycythemia [178] The destruction of the erythropoietic activity in anemic plasma extracts by ashing[179] establishes its organic nature In addition the active principle possesses neither a negative nor a positive electrical charge Boiled plasma extracts passed through both Dowex 50 x8 NH_4^+ and Dowex 1 x10 $COOH^-$ ion exchange resin columns have been found to retain their full erythropoietic stimulatory capacity The materials eluted from both columns were completely devoid of any erythropoietic activity

Early studies[171] suggested that the thermostable plasma erythropoietic factor was ether soluble Boiled anemic plasma extracts precipitated with trichloroacetic acid were inactive whereas precipitation with perchloric acid did not alter the recipients erythropoietic responses Since the effect on any retained protein material in these extracts would be the same with either agent the apparent explanation for this finding was that the ether extraction process used to eliminate the excess trichloroacetic acid also removed the stimulating factor The solubility in ether of the thermostable plasma factor has now been confirmed [180]

Plasma from rabbits rendered anemic by the administration of phenylhydrazine was procured and divided into two portions One aliquot was extracted five times with a volume of reagent ether equal to the original volume of the plasma The ether phase was placed under reduced pressure to remove the ether and reconstituted with distilled water The water phase was boiled and precipitated with perchloric acid as previously outlined The remaining portion of the original anemic plasma was then subjected to the later procedures and divided into two parts One portion was assayed as such The other was extracted five times with an equal volume of reagent ether and evaporated to complete dryness Reagent ether was then readded

in an amount equivalent to twice the original volume of the plasma filtered and the ether was removed under reduced pressure A small amount of greenish oily material remained following this last step and was reconstituted to the original volume of the plasma by the addition of distilled water

The results of this study are shown in Figures 11 and 12 Erythropoietic stimulation was evident in the animals injected with the ether extract of whole "anemic plasma the boiled "anemic" plasma extract and the ether soluble portion of the latter The

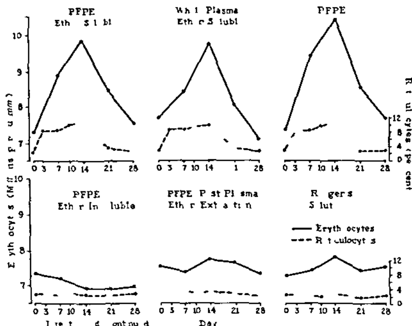


FIGURE 11 —Erythrocytosis and reticulocytosis in rats injected with boiled or "protein free plasma extracts (PFPE) from anemic rabbits the ether soluble fraction of these extracts and ether extracts of otherwise unmodified "anemic plasma without evidence of erythropoietic stimulatory activity in the boiled filtrates of the materials remaining after extraction with ether The increases in red cell and reticulocyte counts were not associated with significant changes in the hemoglobin or hematocrit levels Average counts of twelve rats injected with the ether soluble and ether insoluble portions of the "anemic plasma extract and of six rats in each of the other groups (From Linman J W Bethell F H and Long M J Studies on the nature of the plasma erythropoietic factor(s) *J Lab and Clin Med* *51* 8 16 1958 Reprinted by permission)

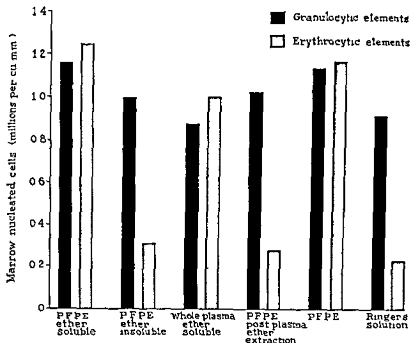


FIGURE 12 Myeloid erythrocytic hyperplasia at the end of a two week injection period in recipients of the ether soluble fractions of a boiled anemic plasma extract (PFPE) and whole anemic" plasma The evidence of erythropoietic stimulation in the peripheral blood of these animals is depicted in Figure 11 Average counts of four rats in each group (From Linman J W Bethell F H and Long M J Studies on the nature of the plasma erythropoietic factor(s) *J Lab and Clin Med* *51* 8 16 1958 Reprinted by permission)

other groups exhibited no alterations in erythropoiesis These observations would certainly appear to establish the ether solubility of the heat stable erythropoietic factor The presence of stimulatory activity in the material extracted twice with ether including evaporation to complete dryness interposed between the two extractions and the absence of activity in the other fractions obtained by this procedure are findings of considerable import in this regard They eliminate the possibility of this factor being water soluble and ether insoluble but present at the ether water interface or carried over in the small amount of water contained in the ether phase following the initial extraction

The removal by ether of all demonstrable activity from previously unmodified "anemic" plasma with identical responses in the recipient animals given this material and the boiled extracts not treated with ether contributes additional experimental support for the ether solubility of the thermostable factor It should be noted however that the failure to detect activity in the plasma remaining after ether extraction does not exclude the presence of a water soluble erythropoietic factor In the above experiment this material was subjected to prolonged boiling prior to testing This procedure has been shown to inactivate the agent in anemic plasma which is capable of stimulating hemoglobin synthesis (see page 75) In fact subsequent studies have shown that the ether insoluble fractions of active plasmas do enhance the incorporation of iron 59 in recipient rats when assayed in an otherwise unmodified state or after boiling for only short periods of time

The above observations have been confirmed on plasmas obtained under a variety of experimental and clinical conditions with identical responses in normal rats given the ether soluble fractions of otherwise untreated plasmas boiled plasma extracts or lyophilized specimens Thus solubility in ether at neutral pH or after acidification of the parent plasma can be added to the other chemical characteristics of the thermostable factor which induces in normal rats the unique erythropoietic response previously described This humoral agent is also soluble in chloroform Although positive identification of this erythrocytogenic substance has not yet been made the available data would appear to exclude many known biologically active compounds and indicates that it is most likely a lipid In this respect the similarities between the chemical physical and physiologic properties of batyl alcohol and the ether soluble thermostable plasma erythropoietic factor are such that a close relationship seems likely (see Chapter VI)

APPARENT DISCREPANCIES IN EXPERIMENTAL OBSERVATIONS

It would seem pertinent at this time to review the often strikingly variable and seemingly divergent data that have been reported by different investigators studying the humoral erythro

poietic regulatory mechanism The failure until recently, of many workers to confirm the presence of erythropoietic stimulatory activity in anemic plasma or serum has already been mentioned The majority of these experiments however involved only a single or at most two or three injections of the test material into normal animals The need for multiple injections of relatively large amounts of plasma or serum undoubtedly explains these negative results

The findings in regard to the nature of the factor or factors responsible for the stimulatory activity of certain plasmas and sera are more disturbing Variability in experimental observations has been apparent since Carnot and Deflandre first hypothsized the existence of a humoral factor They reported loss of erythropoietic activity when anemic serum was heated to 56° C [106] whereas Muller [111] a few years later noted complete retention of activity after heating to this temperature The controversy as to the thermostability of the humoral erythropoietic factor still persists Although the demonstration of erythropoietic stimulatory activity in boiled extracts of anemic plasma by Borsook and co workers[1 0] and Gordon and his associates[1 1] has been confirmed repeatedly by many investigators [1 8, 130, 131, 143, 149, 15, 163, 171, 17, 179, 180, 197] some have failed to find activity in heat denatured materials [149, 194] In more recent studies Stohlman and Brecher[199] have reported that 70 to 90 per cent of the erythropoietic activity present in plasma from donors with hypoxic hypoxia was destroyed by heating to 100°C for ten minutes Heating for 60 minutes did not produce a further decrease in the stimulatory effect They noted retention of activity in unconcentrated plasma boiled for five minutes but not after 30 or 60 minutes Similar findings in regard to the effect of prolonged boiling on plasma erythropoietic activity have been noted by other workers [131, 195, 197] Gurney Jacobson and Goldwasser [200] using the uptake of iron 59 in erythrocytes of hypophysectomized rats as an index of activity have described complete loss after heat denaturation of the erythropoietic stimulatory effect contained in active unmodified plasmas

Although the concensus is that the erythropoietic agent in active plasmas is nondialyzable some have found it to pass through

a semipermeable membrane[181, 201] A number of investigators[191, 201, 207] have reported that the erythropoietic stimulatory effect of active plasmas or sera is abolished by exposure to oxygen and it has been suggested that certain failures to obtain evidence of accelerated erythropoiesis may have been due to aging or aeration of the plasma or serum However retention of activity in "anemic" plasma despite equilibration with 100 per cent oxygen for 24 hours has recently been described [5] This finding supports the many other studies which have shown that anaerobic manipulation of the test materials is not a prerequisite for the demonstration of erythropoietic stimulation in the recipient

Because of these widely differing observations it is easily understandable that many theories and diverse views have arisen as to the chemical structure of the plasma erythropoietic factor There exists evidence which supports the contention that a lipid agent is involved in the humoral control of erythropoiesis Tei[116] first described such a substance in 1938 and Toha and co workers[181] concluded on the basis of their early experiments that the active factor was possibly a fatty acid ester but not an amino acid purine or sugar Using organic extraction procedures coupled with infrared spectrophotometry Gley and Delor[93, 10] have suggested that the primary erythropoietic factor is a steroid possessing one functional alcoholic and three functional ketonic groups

In contrast to this view however the majority of the workers in this field are of the opinion that the plasma erythropoietic factor is protein in nature Erslev and Lavietes [11] concluded in 1954 that the active agent was attached to or behaved like a serum albumin alpha globulin or beta globulin More recent studies indicate that it is a small molecular weight glycoprotein

Rambach Alt and Cooper[139, 163, 193, 194] have reported that the erythropoietic factor in anemic" plasma is heat stable insoluble in ether migrates on paper electrophores s as a single homogeneous component with a mobility between that of alpha 2 and alpha 1 globulins stains as a glycoprotein has a low isoelectric *point and is not sedimented by centrifugation at 103 000 g for* twenty four hours These investigators found the protein sialic acid hexose as glucose equivalent and glucosamine content of the erythropoietically active material isolated on a diethylaminoethyl

cellulose ion exchange column from anemic rabbit plasma to be 69 3, 15 6 7 7 and 10 0 per cent respectively They conclude that it is an acidic glycoprotein Sialic or neuraminic acid alone was without effect and hydrolytic removal of this substance rendered the mucoprotein erythropoietically inactive

The studies of Gordon and his associates[16 1] have also indicated that the erythropoietic stimulating factor cannot be extracted from boiled plasma filtrates with petroleum ether or ethyl acetate Chromatographic techniques failed to reveal specific qualitative differences between active and control filtrates of rabbit plasma with respect to free amino acids purines pyrimidines sugars and organic acids When the cationic dye toluidine blue was added to boiled plasma filtrates they noted the development of metachromasia in chromatograms of active materials Gordon has also suggested on the basis of these observations that the plasma factor may be either a mucoprotein or associated with such a substance

Slaunwhite Mirand and Prentice [13] have concluded from their studies on the properties of the erythropoietic factor in anemic plasma that it is probably a polypeptide They reported that it is heat stable at pH 5 5 and pH 9 but is inactivated by heat at pH 1 and pH 13 is nondialyzable not extractable with chloroform and is digested by pepsin trypsin and chymotrypsin

Borsook and his co workers[131 195] have also concluded from their observations on the chemical characterization of the erythropoietic factor that it is a mucoprotein They have been unable to separate activity from protein and have found the active agent to be insoluble in ether precipitable by 80 per cent saturated ammonium sulfate and to give a positive direct sialic acid test Although resistant to boiling for five minutes or less a loss of as much as 75 per cent of the activity was noted after boiling for one hour On paper electrophoresis their active fractions gave the same bands but a different density distribution from those of Rambach and his associates [163] Two thirds of the protein and all of the activity in their plasma fraction was confined to one band but these investigators do not believe that the material in this band can as yet be considered homogeneous

Grant and his associates [14] have reported that the erythropoietic activity in boiled filtrates of anemic rabbit plasma can be precipitated with ethanol between concentrations of 60 and 80 per cent Their active fraction obtained in this manner was digestible by proteolytic enzymes and inactive orally Earlier studies from this laboratory [15] indicated that the agent in anemic plasma responsible for an accelerating effect on the P^{32} uptake in DNA of recipient animals settled during ultracentrifugation

As mentioned previously Hodgson Toha and their collaborators originally described data indicating that the erythropoietic factor in anemic plasma was a lipid [181, 01] However on the basis of their more recent studies [1, 6] utilizing iron 59 incorporation and turnover as indices of response these workers now support the view that the active substance is mucoprotein in nature

Van Dyke and Garcia [16] have suggested that the erythropoietic substance detectable in the urine of anemic donors is a polypeptide structure They base their conclusion on observations that the active agent does not pass through a collodion membrane on ultrafiltration is not altered by washing with ether or alcohol withstands boiling and is digested with trypsin Winkert Gordon Piliero and Medici [17] have described studies resulting in the partial purification of the erythropoietic factor in the urine of a five year old boy with hereditary leptocytosis Utilizing a kaolin adsorption technique these workers recovered in the 1M NH_4OH eluate 39 per cent of the original activity at a purity 230 times that of raw urine This purified preparation produced a strong metachromatic reaction with toluidine blue and an absorption peak at 280 millimicrons They conclude that these observations are not inconsistent with the thesis that the erythropoietic factor may be a mucoprotein or an associated substance In contradistinction to the reported electrophoretic characteristics of the active substance in anemic rabbit plasma described by Rambach and his associates [161] Winkert and co workers were unable to detect any material in their erythropoietically active urine fractions that migrated on paper electrophoresis as far as the alpha globulins Hodgson and his associates [18] have reported that the erythropoietic factor in anemic urine contains more than 10 mg of

carbohydrate per 100 mg of protein and also conclude that this agent is a mucoprotein

Thus there exist two opposing views in respect to the nature of the substance in certain "anemic" plasmas and urines which is capable of altering the erythropoietic activity of recipient animals. One school of thought contends with excellent experimental support that the active agent is at least relatively resistant to heating, is nondialyzable, can not be precipitated with perchloric acid but is precipitable with 70 to 80 per cent saturated ammonium sulfate or 60 to 80 per cent ethanol, and is destroyed by proteolytic enzymes. This material is soluble in water but not in ether or other lipid solvents, contains sialic acid, possesses metachromatic properties, and migrates on electrophoresis with a mobility characteristic of the alpha globulins. All of these findings support the contention that this factor is a small molecular weight acidic glycoprotein, a conclusion with which we concur (see page 76). On the other hand, our studies together with those of others indicate that an erythropoietic factor possessing the attributes of a lipid can also be extracted from these "anemic" plasmas or sera. This substance is markedly thermostable, soluble in ether and chloroform, and may be closely related to batyl alcohol, the monoglycerol ether of n-octadecyl alcohol, which has been isolated from yellow bone marrow and shown to possess erythropoietic stimulatory activity (see Chapter VI).

The type of response evoked in animals given erythropoietically active sera, plasmas, or extracts thereof has also varied. The erythrocytosis due to the formation of microcytes without associated augmentation in hemoglobin synthesis which we have repeatedly observed in normal rats injected with boiled filtrates of "anemic" plasmas has not been noted by some investigators who have reported elevations in hemoglobins and hematocrits, increased total red cell volume, and enhanced incorporation of iron 59 in hemoglobin in recipients of heat denatured "anemic" plasma extracts. However, Gordon and his associates[166, 184, 1, 13] have described responses in normal rats injected with boiled plasma filtrates similar to ours. Bonsdorff[13] has also reported a lack of parallelism between the increase in erythrocyte counts and the other periph

eral erythroid determinations in recipients of certain test materials

On the basis of the preceding discussion it is evident that this rapidly expanding area of hematologic research has been characterized by confusion and on occasion by grossly incompatible conclusions It is vital that these divergent observations be explained and reconciled Until this can be done and some semblance of order and uniformity be established in this seemingly chaotic field it is virtually impossible to assimilate the current data and assess their true significance

EVIDENCE FOR THE EXISTENCE OF TWO HUMORAL FACTORS

The widely varying views on the nature and mode of action or physiologic effect of the plasma erythropoietic factor(s) may possibly be explained by the following considerations 1) The type of biologic material studied and the circumstances under which it was obtained 2) Donor and recipient species differences 3) Different methods employed to demonstrate erythropoietic stimulation in the recipient 4) Possible alterations in the active factor induced by the techniques utilized in handling and processing the test materials 5) The existence of more than one humoral erythropoietic factor

The methods used to enhance the donor s erythropoietic activity have been multiple and in addition to their presence in plasma and serum erythrocytogenic substances have been described in urine[185, 201, 18, 221] milk[77, 223] and yellow bone marrow[133] However there is no evidence to suggest that the erythropoietic response to any of these source materials is different nor do the methods used to augment this activity e g bleeding phenylhydrazine or simulated high altitudes appear to affect the nature of the recipient s response Rabbits and rats have been most commonly employed as donors but guinea pigs[110] dogs[110, 224] cattle[133] sheep[76] goats[3] monkeys[1, 7] and man[134, 137, 149, 150, 1, 18, 188, 190, 14, 0, 0, 203, 0, 07, 17, 70, 1, 227, 31] have also been used Thus the observations made with a wide variety of sources and recipients (see page 28) indicate a lack of species specificity and dis

credit the above as explanations for the apparent divergent experimental results Although we have utilized Wistar strain rats as recipients in most of our studies a number of other strains have manifested identical responses

The many means of demonstrating erythropoietic stimulatory activity together with the use of both normal recipients and those with depressed erythropoiesis often make critical comparison of different studies difficult Although the types of assay methods employed are undoubtedly responsible for certain discrepancies in experimental data there is no valid reason to assume that these differences reflect specific defects in the techniques

The question as to whether or not alterations in a single erythropoietic factor might result from the varied procedures that have been used to process plasmas and plasma extracts for testing certainly deserves comment Such changes conceivably could induce a different kind of erythropoietic response in recipient animals However experimental evidence does not support this possibility Ether extracts of whole anemic plasma evoke the same erythropoietic response in normal rats as do boiled and perchloric acid precipitated plasma fractions and the ether extracts thereof This finding would indicate that boiling and treatment with perchloric acid do not alter the type or magnitude of the erythropoietic stimulus contained in boiled anemic plasma extracts Furthermore an enhancing effect on the incorporation of iron 59 in hemoglobin is retained in the ether insoluble fractions of active plasmas Additional evidence against alterations in a single factor can be found in patients with polycythemia vera (see page 159) It consists of the demonstration in the blood of these patients of microcytes with abnormal osmotic behavior which are strikingly similar to those observed in normal rats injected with boiled or ether extracts of polycythemic or other erythropoietically active plasmas For these reasons changes in the chemical and physiologic properties of a single factor by processing procedures would appear to be an unlikely possibility

It has been proposed that the failure of certain heat denatured plasma extracts to evoke an increase in all of a recipient s erythroid values may be related to the potency of the stimulus contained therein However it is difficult to envision a suboptimal

stimulus as affecting some but not all of the parameters that reflect erythropoietic activity On the basis of our current knowledge of the physiology of erythropoiesis it would be more plausible to expect providing all other conditions remained normal a *similar but less marked response* with lesser degrees of stimulation A slight increase in reticulocytes or iron 59 incorporation even though indicative of accelerated erythropoiesis may not be accompanied by detectable elevations in the hemoglobin or hematocrit levels However the magnitude of the reticulocytosis and myeloid erythrocytic hyperplasia together with the erythrocytosis seen in recipients of boiled plasma extracts are such that it would seem very unlikely that a greater but identical stimulus would be needed to augment hemoglobin synthesis Moreover observations on plasmas from donors with severe moderate or minimal anemias have shown that even though the degree of the response in normal rats given the boiled extracts of such materials may differ presumably due to the level of humoral factor activity contained in the parent plasma the *type of response* remains the same Studies on the effect of different doses of boiled "anemic" plasma extracts also support this concept (see page 86) Whereas certain minor discrepancies in the results of otherwise identical experiments may be due to different levels of plasma factor activity in the materials tested it is impossible to reconcile major areas of disagreement by such an explanation

Gordon[1 3] has suggested that the production of microcytes may be partially prevented by the inclusion of extra inorganic iron in the diet of rats receiving active plasma extracts However there is no evidence of hypochromia in the erythrocytes of recipient rats displaying erythromicrocytosis Furthermore it has been shown that active plasmas from donors with phenylhydrazine induced hemolytic anemia or from those given cobalt although capable of stimulating hemoglobin production do not augment the gastrointestinal absorption of iron in recipients [5] Heme synthesis is not impaired in these animals even though greater than normal amounts of iron are actually being utilized Consequently it would seem improbable that after prolonged boiling these same plasmas could alter iron metabolism in such a way as to produce the microcytic response

Experimental evidence indicates that there are at least two plasma erythropoietic factors Our initial research activities dealt with the erythropoietic activity in filtrates of anemic plasma processed by boiling for a total of 30 minutes or more followed by precipitation with perchloric acid Although these extracts induced erythrocytosis reticulocytosis and myeloid erythrocytic hyperplasia when administered to normal rats they failed to augment circulating hemoglobin or red cell mass and were ineffective in enhancing the incorporation of iron 59 in the hemoglobin of recipient animals The response to the same source materials however when tested in the unmodified state or after boiling for

TABLE VII

Effect of Boiling on the Erythropoietic Stimulatory Activity of Phenylhydrazine Induced Anemic Rabbit Plasma as Measured by the Incorporation of Iron 59 in Hemoglobin of Nitrogen Mustard-treated Rats Means of 4 Rats in Each Group ±1 Standard Deviation (From Linman J W Korst D R and Bethell F H Some observations on the stimulation of erythropoiesis by humoral factors Ann New York Acad Sc 77 Art 3 638 649 1959 Reprinted by permission)

Material Tested	*Per Cent Fe-59 RBC Uptake*		
	18 Hours	*24 Hours*	*42 Hours*
Unmodified anemic plasma	16±2	19±4	34±5
Anemic plasma Boiled 5 min	13±2	20±3	35±2
Anemic plasma Boiled 10 min	7±3	10±3	23±4
Anemic plasma Boiled 30 min	7±2	9±2	23±4
Anemic plasma Boiled 45 min	5±2	6±2	25±3
Normal Rabbit Plasma	6±2	9±2	27±5

only short periods of time was characterized by an increase in the erythrocyte counts hemoglobin and hematocrit values and rate of iron 59 incorporation in hemoglobin This observation suggested the presence of an agent in whole "anemic" plasma which augmented hemoglobin synthesis but did not possess the marked thermostability of the factor in boiled extracts

The effect of boiling for varying periods of time on the erythropoietic activity of plasma obtained from rabbits rendered anemic by the administration of phenylhydrazine is shown in Table VII It can be seen that boiling for five minutes did not alter the erythropoietic activity of the plasma as measured by the incorporation of iron 59 in hemoglobin of nitrogen mustard treated rats However boiling for longer periods of time abolished this effect and the responses of animals injected with such materials were comparable to those of rats receiving normal "non anemic" plasma Although inactive by this method of demonstrating an erythropoietic effect the boiled extracts still retained their ability to evoke erythromicrocytosis reticulocytosis and myeloid erythrocytic hyperplasia in normal rats given multiple daily injections The ether or cholorform soluble fractions of active plasmas also failed to alter iron 59 uptake but the material extracted with these lipid solvents induced the same type of microcytic response observed in recipients of boiled plasma filtrates

We believe that these separable effects exerted by erythropoietically active plasmas are due to the presence of two factors which differ in nature and mode of action One effect is characterized by the development in recipient animals of erythrocytosis secondary to the production of increased numbers of small cells reticulocytosis and myeloid erythrocytic hyperplasia without evidence of enhanced hemoglobin production It is related to a heat stable ether soluble fraction of plasma and the agent responsible for this erythropoietic effect is probably a lipid These findings which indicate that a lipid is involved in the control of erythropoiesis are supported by and give apparent meaning to several observations such as the increase in blood lipids that follows acute hemorrhage and the alterations in marrow fat of rabbits induced by bleeding or the administration of phenylhydrazine[33, 34]

The other erythropoietic effect contained in anemic plasmas is manifested in recipient animals by increased hemoglobin synthesis and is detectable by such techniques as the erythrocytic uptake of iron 59 in addition to changes in other parameters which reflect hemoglobin production It is related to a substance which is relatively thermolabile and soluble in water but not in ether or chloroform This agent is nondialyzable resists lyophilization and is not inactivated by exposure to oxygen or freezing However it does not remain active in the frozen state for as long periods of time as does the thermostable ether soluble factor Frozen "anemic plasmas retain their enhancing effect on iron 59 incorporation for as long as two months after which they rapidly lose this property Our other studies in regard to the chemical nature of this erythropoietic substance are in agreement with those of others and support the conclusion that it is a mucoprotein or closely associated with such a substance

The concept of more than one humoral factor concerned with the regulatory control of erythropoiesis is not a new one In 1938 Tei[116] described an erythropoietic substance which resisted boiling and was soluble in ether acetone and alcohol in the serum of animals that had been bled or given phenylhydrazine and concluded that it was probably a lipid In the serum of animals subjected to reduced oxygen tension or fed garlic he reported the presence of another factor which was thermolabile and ether insoluble Tei postulated that it was probably a globulin More recently Gley [09, 10, 3] has also described two factors in the blood of animals subjected to hemorrhage one had the solubility characteristics of a lipid and the other was water soluble He has suggested that the former hematopoietine is the primary factor which controls the multiplication of erythrocytic precursors and is possibly a trioxomonoalcoholic sterol whereas the latter hematostimuline has properties closer to the protein group and may exert an indirect effect

The apparent disparity in many experimental observations on the nature and type of response induced by certain plasmas sera and urines can be explained on the basis of two factors with different chemical and physiologic properties Some variance in findings would then be expected depending upon the type of material

studied and the assay method employed The demonstration of erythropoietic stimulation in recipients of active plasmas or extracts by techniques utilizing as an index of activity the incorporation of iron 59 in hemoglobin or measurements of hemoglobin hematocrit or total blood volume would depend upon the presence in the materials tested of the relatively thermolabile factor which enhances hemoglobin synthesis These methods would not yield evidence of the production of increased numbers of microcytes which accompanies the administration of the heat stable factor alone

Unmodified active plasmas give positive results regardless of the type of assay method used Gordon [12] has described increases in both the hemoglobin determinations and erythrocyte counts in normal rats given multiple injections of unmodified anemic rat plasma However when the plasma was boiled for 15 minutes prior to testing the recipients developed erythrocytosis reticulocytosis and myeloid erythrocytic hyperplasia but without associated increase in their hemoglobin levels We have noted similar findings with comparable test materials and this method of demonstrating erythropoietic activity It may be concluded from these observations that the combined effects of both the thermostable and relatively thermolabile factors contained in whole "anemic" plasma result in the formation of increased numbers of normal erythrocytes Therefore the recipients of whole plasma manifest an increase in all erythroid determinations The different response observed in recipients of heat denatured anemic" plasma extracts would then appear to be due to the selective removal by boiling of the relatively thermolabile factor which stimulates hemoglobin synthesis with retention of the thermostable ether soluble agent

It should be noted that boiling the test plasma for five to ten minutes or placing it in a boiling water bath for similar relatively short periods of time will not completely inactivate the relatively thermolabile factor This manner of preparing plasma extracts for testing has been much more generally used than has prolonged boiling over a direct flame These materials would possess an erythropoietic stimulatory effect attributable to the combined activities of both humoral factors and would appear to explain in

essentially every instance the reports of increases in hemoglobin and or hematocrit red cell volume and iron 59 uptake in erythrocytes of recipients given heat denatured plasma extracts

Certain other discrepancies in experimental data may be similarly explained Thus the majority of the studies supporting the protein nature of the plasma erythropoietic factor have used radioisotopic techniques as a measure of response whereas those favoring a lipid have employed changes in such parameters as the erythrocyte or reticulocyte counts Recent observations on the loss of activity after boiling have for the most part been based on the uptake of iron 59 in recipient animals Failure of the ether soluble fractions of active plasmas to enhance the incorporation of iron 59 in recipient animals with retention of this effect in the ether insoluble portions has been construed as a failure to confirm the findings of an ether soluble factor There are many other examples too numerous to mention It may be concluded therefore that the methods of demonstrating activity and of processing plasma serum or urine for testing do play important roles in determining the erythropoietic response observed in recipient animals However the variable results that have been described cannot be attributed to defects in the techniques employed Instead they appear to be due to the presence in erythropoietically active materials of two factors with different chemical and physiologic characteristics

MODES OF ACTION OF THE PLASMA FACTORS

The precise modes of action of the plasma erythropoietic factors have not yet been clearly defined Certain speculations may be made but the potential hazards associated with conclusions based on experimental observations of the response evoked by erythropoietic factors in another living organism should be stressed and must be constantly borne in mind

Erythropoiesis involves not one but a number of physiologic processes (see Chapter I) which together result in the formation of the hemoglobin containing erythron Although their relative complexity and apparent inter relationship in the normal state are recognized erythropoiesis can be divided into the following four

phases 1) Differentiation of the multipotential myeloid reticulum cells into erythrocytic precursors 2) Intramedullary multiplication or proliferation of erythrocytic elements 3) Maturation of erythrocytic precursors 4) Synthesis of hemoglobin Since the end product the erythrocyte is ordinarily confined to the circulation quantitative and qualitative alterations in erythropoietic activity are readily detectable However the specific site of action or type of effect exerted by materials capable of inducing such measurable changes in the peripheral blood of recipient animals can only be inferred from the observable response Thus the demonstration of an increase in circulating hemoglobin red cell mass or incorporation of iron 59 in hemoglobin while clearly indicative of enhanced hemoglobin formation does not delineate which of the physiologic processes comprising erythropoiesis that has been stimulated

In the presence of an intact myeloid reticulum and sufficient amounts of the various substances required for the formation of the normal erythrocyte erythropoiesis proceeds in an orderly manner and superficially would appear to be under a single regulatory mechanism Replacement of myeloid erythrocytic elements that have matured and been released into the circulation by diversion of reticulum cells into red blood cell production cellular division and progressive maturation would seem to occur concomitantly with hemoglobin synthesis Upon closer scrutiny however such an assumption does not appear valid or justified

Many experimental models have been devised in an attempt to elucidate the intramedullary stages of red cell development [38-45] The difficulties associated with such studies are evident and there remains much to be learned but a number of observations relevant to the present discussion have emerged The first step in erythropoiesis consists of differentiation of the myeloid reticulum cells into erythrocytic precursors This process must involve only one of the products of a mitotic division in order to prevent depletion of the primitive pluripotential cells There then follows a period which is characterized by progressive maturation and proliferation Multiplication of erythrocytic precursors occurs chiefly if not exclusively by homeoplastic mitotic division i e one cell gives rise to two cells with the same characteristics as the parent

cell It is not possible to specifically relate cellular division and maturation The number of mitoses increases with differentiation and the more mature mitotic cells contribute perhaps 80 to 90 per cent of the proliferative activity [44] Furthermore quantitative marrow studies demonstrate disproportionately more mature than primitive nucleated red cell precursors and are incompatible with a single or comparable number of cellular divisions occurring between each succeeding recognizable stage of erythrocytic differentiation Finally there is reached a degree of maturity beyond which further multiplication does not take place even though cytoplasmic maturation continues

The synthesis of hemoglobin is a complex process, our knowledge of which has been greatly advanced by newer isotopic cytochemical and tissue culture techniques It is dependent upon the availability of a number of enzymes and metabolic building blocks most notably iron porphyrin and protein (see Chapter I) The production of hemoglobin is intimately associated with the differentiation and maturation of myeloid erythrocytic elements It may be reasonably assumed on the basis of existent data that the physiologic activities of the most primitive erythrocytic precursors are essential for the ultimate formation by later forms of the actual hemoglobin molecule Therefore the synthesis of hemoglobin would appear to result from a continuous sequence of events which is initiated in the rubriblast and continues through the reticulocyte stage of maturation Accepting the monophyletic theory of blood formation it is equally logical to assume that the rubriblast possesses the inherent capacity to synthesize hemoglobin and providing all other conditions are favorable including a uniform rate of mitotic activity that each such cell is the progenitor of a preordained quantity of hemoglobin evenly divided in a given number of mature erythrocytes

Being cognizant of the existent voids in our knowledge it would still seem essential to explore the possible modes of action of the humoral erythropoietic factors The responses observed in recipients of erythropoietically active materials could conceivably result from one or more of the following four mechanisms 1) Augmentation in the amount of hemoglobin synthesized by already differentiated red cell precursors 2) Decreased intramed

ullary maturation time 3) Increased diversion of the primitive multipotential reticulum cells into erythrocyte production 4) Increased erythroblastic cellular division or mitotic activity

In view of the multiplicity of the physiologic processes involved and the fact that hemoglobin synthesis appears to be dependent on a progressive series of metabolic activities initiated in the rubriblast it would seem quite unlikely that a stimulus could be accorded to *relatively mature myeloid erythrocytic elements* which would result in the formation of significantly increased quantities of hemoglobin. Although a number of substances have been found to increase the incorporation of labeled amino acids into the protein of reticulocytes these materials do not exert an *in vivo* erythropoietic effect [16, 18] In addition Jandl and his associates[153] have shown that neither hypoxia nor erythropoietically active plasmas enhance the *in vitro* incorporation of iron into heme by reticulocytes. Acceleration in the rate of hemoglobin synthesis would also appear improbable. It is evident that under conditions of increased need reticulocytes are released into the circulation earlier than would occur normally. However many studies such as those of Alpen and Cranmore [17] indicate that the period of time required for intramedullary development and maturation to this stage or at least to postmitotic forms remains constant regardless of the total output of erythrocytes or hemoglobin by the marrow. Furthermore the myeloid erythrocytic hyperplasia in recipients of active plasmas precludes a decrease in generation time as the sole explanation for the responses observed. It may be concluded that the synthesis of hemoglobin is intimately and inseparably related to the maturation of marrow erythrocytic precursors. These two aspects of erythropoiesis apparently proceed at a relatively fixed rate unaffected by the humoral factors and predetermined perhaps by the nutritional and hormonal environment

The possible effects of increased erythroblastic cellular division and enhanced reticulum cell erythrocytic differentation remain to be considered. It would seem improbable that accelerated mitotic activity of differentiated erythrocytic precursors could augment hemoglobin production. On the other hand it is readily apparent that an *increase in the number of erythrocytic precur*

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cursors undergo during maturation is subject to the regulatory control exerted by the thermostable ether soluble plasma factor

It is often desirable and certainly more convenient in clinical and investigative medicine to attribute a response such as an increase in erythrocyte production to a single agent or effect However there is no compelling support for the contention that hemoglobin production and erythroblastic cellular division must be responsive to a single stimulus or that an increase in cellular division must necessarily follow augmentation in hemoglobin synthesis or *vice versa* There are well documented instances where this does not occur and examples of disparity between these two aspects of erythropoiesis are fairly numerous These include among others the lack of parallelism between erythrocyte and hemoglobin production in hereditary leptocytosis and chronic hemorrhagic or iron deficiency anemia and the increase in hemoglobin content of the macrocytes in pernicious anemia

In vitro tissue culture techniques have shown that the multiplication and maturation of marrow cells may be dissociated Although maturation which is accompanied by hemoglobin synthesis and the multiplication of myeloid erythrocytic precursors takes place concomitantly in vivo conditions that favor proliferation in tissue culture do not necessarily favor maturation Conversely culture techniques most conducive for maturation or differentiation are not compatible with optimal rates of multiplication [3,5,53,54] Other observations also indicate that the proliferation and maturation of erythrocytic elements are separable and distinct physiologic processes Recent studies by Thomas and Lochte [55] provide an example of such support These workers have found that the addition of vitamin B_1 to cultures of erythrocytic marrow cells from patients with pernicious anemia permitted proliferation of these cells due to its effect on DNA synthesis but did not increase heme production It has also been suggested on the basis of experimental data [6] that the decreased erythropoiesis seen in patients given therapeutic doses of nitrogen mustard is due to impaired mitotic activity with fewer cells reaching maturity and does not reflect a specific abnormality in hemoglobin synthesis

sors differentiated from the primitive dividing cells would if all other conditions remained unchanged augment hemoglobin production Consequently this mechanism can be assumed to be responsible for the enhanced hemoglobin synthesis demonstrable in recipients of active plasmas However alterations in the number of erythroblastic cellular divisions cannot be dismissed as inconsequential or of minor importance in the regulation of erythropoiesis

Increased mitotic activity of erythrocytic precursors is evident cytologically in the marrows of experimental animals and human subjects with erythroid hyperplasia secondary to hemorrhage hemolysis or decreased arterial oxygen saturation and in recipients of plasmas from such donors The intimate association of cell proliferation with the synthesis of desoxyribose nucleic acid (DNA) has also been utilized to study changes in mitotic activity The incorporation of radioactive phosphorous into the DNA molecules of bone marrow cells has been shown to accurately reflect their rate of cellular division and is increased by hypoxic hypoxia [50] and by the administration of erythropoietically active plasma extracts [163] Therefore it is important to give attention to the possible relationship between the production of hemoglobin largely a cytoplasmic function and mitotic activity which is basically nuclear

In the past the erythropoietic effect of certain plasmas and sera has been ascribed by most investigators to only one substance which was assumed to stimulate all of the physiologic processes which encompass erythropoiesis More recently it has been proposed that the humoral erythropoietic mechanism acts only to initiate red cell production by inducing reticulum cell erythrocytic differentiation and that subsequent proliferation and maturation occur at fixed rates independent of the humoral stimulus [1] We concur with this theory insofar as the mode of action of the relatively thermolabile plasma erythropoietic factor which appears to be a mucoprotein is concerned However our studies together with a number of other observations do not support the thesis that the multiplication of nucleated erythrocytic precursors is unaffected by the humoral stimulus Instead they suggest that the number of cellular divisions which the erythrocytic pre

TABLE VIII

DISTRIBUTION PATTERN OF MYELOID ERYTHROCYTIC PRECURSORS IN RECIPIENTS OF RINGER'S SOLUTION (CONTROLS) AND THE THERMOSTABLE ETHER SOLUBLE PLASMA ERYTHROPOIETIC FACTOR (STIMULATED ANIMALS)

		Rubriblasts	*Prorubricytes*	*Rubricytes*	*Metarubricytes*
Controls (Average of 64 rats)	Cells per cu mm	3 845	8 960	40 102	241 509
	Ratio	1	2	10	63
Stimulated Animals (Average of 42 rats)	Cells per cu mm	39 540	91 861	304 030	697,551
	Ratio	1	2	8	18
Ratio between Control Animals and Stimulated Animals		1 10	1 10	1 8	1 3

the metarubricytes many of which are probably no longer capable of undergoing mitotic division This observation together with the failure to detect evidence of enhanced hemoglobin synthesis in recipients of these anemic" plasma extracts rules against increased reticulum cell erythrocytic differentiation as a physiologic action of the erythrocytogenic agent contained therein Since it appears improbable that hemoglobin synthesis can be augmented other than by stimulating rubriblast formation from the pluripotential reticulum cells increases in hemoglobin and hematocrit values should have been evident if this had occurred Furthermore the ratio between immature and mature nucleated red cells would be expected to remain constant if their increase did not reflect a greater than normal number of mitotic divisions

The fact that the ratio between the various morphologic stages of erythrocyte development can be changed provides additional evidence that the number of homeoplastic cellular divisions occurring during their maturation is not predetermined and unalterable even though the total generation time appears to be constant irrespective of the presence or absence of accelerated erythropoi

Swann[57, 58] has recently reviewed the complex mechanism of cell division and its control He emphasized the antagonism between this process and differentiation and concluded that growth *per se* is not in any way a direct trigger for division It is not unreasonable then to postulate that the seemingly diverse physiologic processes of hemoglobin synthesis and the proliferation of myeloid erythrocytic precursors may be under the regulatory control of separate humoral factors The experimental observations on the erythropoietic effects of active plasmas and plasma extracts described in the preceding pages support such a theory The apparent mode of action of the relatively thermolabile plasma erythropoietic factor is to induce erythrocytic differentiation of the primitive reticulum cells or hemocytoblasts with a resultant augmentation in hemoglobin synthesis The thermostable ether soluble plasma factor appears to stimulate cellular division of already existent nucleated erythrocytic elements

An increased rate of erythroblastic proliferation would be expected to eventuate in the formation of a greater number of cells even in the absence of a comparable stimulus to hemoglobin synthesis i e augmented reticulum cell erythrocytic differentiation or a shortened maturation time Under these conditions however it may be logically surmised that the new erythrocytes would be small and contain collectively the same amount of hemoglobin as the normal sized cells that would have been formed from the erythrocytic precursors involved had fewer cellular divisions occurred Although the duration of the mitotic process is undoubtedly constant and fixed a decrease in the intermitotic interval or resting stage would permit more mitoses in a given period of time This mechanism is apparently responsible for changes in the rate of cellular proliferation regardless of the tissue involved[40] The unique peripheral erythroid response in normal rats injected with boiled or ether extracts of active plasmas is compatible with such an erythropoietic effect

The marrow findings in the recipients of the thermostable ether soluble factor also support the above hypothesis As shown in Table VIII the myeloid erythrocytic hyperplasia involves all recognizable nucleated red cell elements but with a disproportionately greater increase in the more immature forms than in

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etic activity Some reports indicate a slightly greater increase in immature than mature nucleated erythrocytes in the marrows of experimental animals and human subjects with hypoxic and certain types of anemic hypoxia [9] However most studies have shown that the myeloid erythrocytic hyperplasia which accompanies these conditions involves a roughly proportional increase in all forms with a relatively constant ratio between the four recognizable morphologic stages of development [39, 1, 60] This observation has been interpreted as evidence that maturation and multiplication proceed at a fixed rate Data on the effect of the thermostable plasma erythropoietic factor do not support this conclusion The parallel increase in both humoral factors demonstrable in these hypoxic states would seem a more likely explanation for the mechanism by which this ratio is maintained

In most instances we have employed daily injections of anemic plasma extract reconstituted to the original volume of the parent plasma in doses equivalent to 2 per cent of the recipient s body weight per day This quantity has produced definite evidence of erythropoietic stimulation in normal rats and has been used in order to maintain uniformity and allow comparison of the activity in different plasmas However studies have been carried out to determine the erythrocytogenic effect of varying amounts of an anemic plasma extract [173] The results of these experiments also support the hypothesis that the mode of action of the thermostable plasma factor is to increase erythroblastic cellular division

Groups of rats were given daily subcutaneous injections over a four week period of a boiled extract of anemic plasma in amounts equal to 0 5 1 0 1 5 2 0 2 5 and 3 0 per cent of their body weights respectively It was noted that daily doses greater than 2 per cent of the recipient s body weight failed to induce a significantly greater degree of erythropoietic stimulation On the other hand amounts as small as 0 5 per cent per day were capable of evoking reticulocytosis and erythrocytosis in normal rats but the evidence of erythropoietic stimulation in these animals was delayed in onset It appeared progressively sooner in the other groups as the daily dosage was increased Even so the erythrocyte and reticulocyte counts reached comparable plateaus and

were only slightly less marked at the end of four weeks in the animals receiving 0 5 per cent of their body weight per day than in the rats injected with larger quantities of the plasma extract Femoral marrow studies at the time the injections were terminated showed a comparable degree of erythrocytic hyperplasia in all animals regardless of the total amount of anemic plasma extract they had received

These findings suggest that the responses induced in normal rats by the erythropoietic stimulating factor in boiled anemic plasma extracts is determined not only by the daily dose but by the length of the injection period The time required for maximal stimulation to develop appears to vary inversely with the quantity of the factor administered In other words a specific amount of this factor apparently evokes a certain degree of erythropoietic stimulation in the intact rat whether it be given in a relatively large number of small doses or in greater daily amounts over a shorter period of time

If the thermostable ether soluble factor acted to divert a greater number of primitive undifferentiated myeloid reticulum cells into erythrocyte production the increase in circulating erythrocytes would then seem to be limited only by the available intravascular space Under these conditions the response to small amounts should always lag behind that induced by larger quantities However if the ultimate erythropoietic response was related to the number of pre treatment marrow red cell precursors without a significant increase in reticulum cell erythrocytic differentiation over that which would occur normally a definite ceiling would be anticipated The length of time required for this to be achieved would depend on the dosage given This concept is also supported by the constancy of stimulated recipient s marrow nucleated cell counts whether they are injected for two four or six weeks The rapid return to normal after the injections are stopped rules against possible storage and threshold effects

Since the small cells produced in the normal rat injected with the thermostable plasma factor apparently possess impaired viability this must be considered as a possible determinant of the response observed On the basis of the rapidity of the restoration of normal red cell counts and Price Jones curves after the injec

tions are stopped (Figs 7 8 and 11), it is reasonable to estimate a survival time for the microcytes of approximately 15 days Consequently the impaired viability of these cells cannot be assumed to affect the type or magnitude of the response observed in short term experiments of two weeks or less When the injections are continued for longer periods of time it is necessary to postulate some increase in hemoglobin production in order to maintain the normal oxygen carrying capacity of the blood The stability of the hemoglobin and hematocrit values indicates that this does occur and is probably the result of enhanced endogenous formation of the erythropoietic factor which stimulates hemoglobin synthesis Under these experimental conditions i e injection periods of three or more weeks there must exist accelerated rates of both production and destruction with the former predominating until providing the stimulus is continued long enough equilibrium is re established at a higher level Such a mechanism could play an important role in the development of the plateaus in the erythrocyte and reticulocyte counts evident in long term experiments Even so if the thermostable factor acted in some manner other than to accelerate cellular division of differentiated erythrocytic elements the responses to varying doses would again be expected to bear a constant relationship to each other and greater myeloid erythrocytic hyperplasia would be anticipated with larger doses

Erslev [1 5] has recently described experiments concerning the effect of anemic hypoxia on the cellular development of nucleated red cells Normal rabbits were bled kept anemic for twenty hours and then reinfused with the previously removed blood A characteristic reticulocyte response followed and reached its maximum in three days despite the absence of continued anemic hypoxia The reticulocytosis was not diminished by placing the animals in an atmosphere of 65 per cent oxygen immediately after the anemia was corrected and was not significantly increased by the continued presence of anemia When mitotic division was completely but reversibly arrested by the administration of colchicine during the period of anemic hypoxia the reticulocyte response was delayed for twenty four to forty eight hours but was otherwise of characteristic magnitude Colchicine given imme

diately after the period of anemia modified the response which began as usual but disappeared twenty four hours later Erslev has concluded from these observations that the hypoxic stimulus initiates erythropoiesis by accelerating the differentiation of myeloid reticulum cells into rubriblasts but that subsequent multiplication maturation and hemoglobin synthesis proceed at fixed rates independent of the hypoxic stimulus Although entirely compatible with this thesis these data may be interpreted differently and are not necessarily incompatible with the contention that the proliferation of erythrocytic precursors is also under humoral control

Some reports indicate that the plasma erythropoietic activity of anemic donors can be rapidly abolished by transfusions [90, 19] thus suggesting that the biologic action is short lived These observations support Erslev s conclusions However if the level of plasma activity is dependent on marrow utilization such an interpretation may not be justified In this respect it has been shown that the plasma from anemic patients with intact hyperactive marrows is less active than that from patients whose marrows are hypoplastic or otherwise unable to respond to the humoral stimulus [90]

Recent studies by Stohlman and Brecher [61] also indicate that the level of plasma erythropoietic activity reflects the balance between production and utilization Using intact rats which had been subjected to hemorrhage or simulated high altitude as donors these investigators noted enhanced erythropoietic activity of these plasmas when assayed in other rats This stimulatory effect reached a peak in twelve to twenty four hours and had diminished markedly at forty eight hours Increased activity was no longer demonstrable after ninety hours When the donors erythrocyte production was depressed by x irradiation prior to the application of the hypoxic stimulus their plasmas were still erythropoietically active ninety hours later Since the immediate effect of both colchicine and ionizing radiation is to inhibit mitosis the delayed *response noted by Erslev in rabbits given colchicine during the period* of anemia may have been due to persistently elevated levels of plasma factor activity resulting from the marrow s temporary inability to respond to the stimulus It is equally reasonable to

tions are stopped (Figs 7, 8 and 11), it is reasonable to estimate a survival time for the microcytes of approximately 15 days Consequently the impaired viability of these cells cannot be assumed to affect the type or magnitude of the response observed in short term experiments of two weeks or less When the injections are continued for longer periods of time it is necessary to postulate some increase in hemoglobin production in order to maintain the normal oxygen carrying capacity of the blood The stability of the hemoglobin and hematocrit values indicates that this does occur and is probably the result of enhanced endogenous formation of the erythropoietic factor which stimulates hemoglobin synthesis Under these experimental conditions i e injection periods of three or more weeks there must exist accelerated rates of both production and destruction with the former predominating until providing the stimulus is continued long enough equilibrium is re established at a higher level Such a mechanism could play an important role in the development of the plateaus in the erythrocyte and reticulocyte counts evident in long term experiments Even so if the thermostable factor acted in some manner other than to accelerate cellular division of differentiated erythrocytic elements the responses to varying doses would again be expected to bear a constant relationship to each other and greater myeloid erythrocytic hyperplasia would be anticipated with larger doses

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A similar explanation applies to the findings of Alpen and Cranmore [19] which indicate that the number of cellular divisions occurring from the time of entry into the erythrocytic series to completion of maturation is the same in both bled and unbled dogs Since hemorrhage is followed by an increase in the activity of both plasma factors more erythrocytic precursors would be derived from the multipotential reticulum cells Greater amounts of the thermostable factor would then be needed to maintain the uniform number of cellular divisions apparently needed for the formation of cells which are normal in size and hemoglobin content for the species involved In this regard the increased mitotic figures and enhanced incorporation of radioactive phosphorus into DNA demonstrable in the marrow in certain anemic and hypoxic states (see page 82) probably reflect the presence of more mitotable cells rather than any increase in the number of divisions over those which would occur normally during the maturation process

The possibility that the hematologic phenomena ascribed to the thermostable ether soluble plasma factor may be the result of hemolytic rather than true erythropoietic stimulatory properties must be excluded The failure of this agent to enhance hemoglobin synthesis is not in itself sufficient evidence to warrant the assumption that it cannot constitute a basic stimulus to erythropoiesis The hemoglobin content of the erythrocytes is essential for the maintenance of normal cellular metabolic activity However it is equally important that they be of the appropriate size and shape for the successful completion of their physiologic function and alterations in erythropoiesis are not necessarily reflected by changes in hemoglobin production Furthermore experimental data would appear to eliminate hemolysis as an explanation for the erythrocytic responses observed in normal rats given the thermostable or ether soluble fractions of active plasmas

The reticulocytosis and myeloid erythrocytic hyperplasia are compatible with hemolysis but the other findings in recipients of these plasma extracts are not The absence of anemia does not in itself exclude hemolysis A discernible reduction in circulating hemoglobin or red cell mass occurs only when erythrocytogenesis fails to keep pace with the peripheral loss Increased myeloid

conjecture that the time required for utilization by the marrow of the factors elaborated in response to the anemia may have played a role in the other observations described by Erslev there by explaining the fact that the reticulocytosis was not dependent on the continued *presence of the hypoxic state or influenced by* hyperoxia which apparently depresses the endogenous formation of the factors

The criticism has been advanced that the production of microcytes without augmentation in hemoglobin synthesis depicts an abnormal erythropoietic response and could not therefore represent the true mode of action of a substance involved in the physiologic control of erythropoiesis However when one considers the experimental circumstances under which this response is elicitable it does not appear unlikely The type of erythropoietic response observed in normal recipient animals given the thermostable or ether soluble fractions of active plasmas occurs in the absence of any evidence of accelerated hemoglobin production The unequivocal myeloid erythrocytic hyperplasia in these animals establishes the presence of increased erythroblastic cytokinesis Consequently the formation of microcytes would be expected The abnormal osmotic behavior of the microcytes formed in normal rats injected with the thermostable factor probably reflects their altered size and/or shape rather than a specific physiologic effect on cell viability

A situation involving isolated enhancement in the endogenous activity of the thermostable plasma erythropoietic factor comparable to the above experimental conditions would appear highly improbable An associated increase in the relatively thermolabile factor which exerts its effect on hemoglobin synthesis can however be logically hypothecated and is supported by experimental evidence We have yet to study an active plasma regardless of the experimental or clinical conditions under which it was obtained that has not possessed activity attributable to both factors Under these circumstances the parallel increase in both factors augments hemoglobin synthesis and cellular division The production of increased numbers of normal sized cells with maintenance of a steady ratio between the different marrow nucleated erythrocytic precursors results

be necessary as a prerequisite for the maintenance of stable hemoglobin and hematocrit values to postulate the presence of definite augmentation in hemoglobin production Although this radioisotopic technique of demonstrating enhanced hemoglobin synthesis is relatively insensitive to minor changes the myeloid erythrocytic hyperplasia and reticulocytosis in these recipient animals are marked and occur promptly There would appear to be little doubt that hemolytic activity of this degree whether compensated or not, would be reflected by enhanced iron 59 incorporation in hemoglobin Since the oxygen carrying capacity of the blood is maintained without evidence of enhanced hemoglobin synthesis the microcytes produced in response to this particular stimulus cannot be considered nonfunctional Their individual hemoglobin content is reduced but the total circulating hemoglobin remains normal Therefore the persistent reticulocytosis observed in these recipient rats probably reflects the increased number of cells reaching this stage of development

One other possible effect of the thermostable ether soluble factor deserves comment but it too would seem excludable by experimental data The erythrocytosis without change in the hemoglobin or hematocrit determinations could be explained by fragmentation of circulating red cells [*] The lack of poikilocytosis the size of the microcytes and the uniformity of the small cells giving rise to a double peaked Price Jones curve (Fig 8) are against such an occurrence Moreover such a mechanism could not be correlated with the reticulocytosis and myeloid erythrocytic hyperplasia unless the viability of the fragments resulting from erythrocytic cytoclasis was markedly impaired It would then be possible to reconcile all the peripheral blood and marrow findings However under these conditions enhanced iron 59 incorporation in hemoglobin should of necessity be easily demonstrable

The existence of two humoral factors with different natures and modes of action is in agreement with and apparently explains a number of other observations It is of interest in this regard that heat denatured serum has been found to increase the oxygen consumption of marrow cells *in vitro* but does not exert an effect on heme synthesis The latter was reported to be enhanced by unheated serum [79] In these studies serum heated for thirty minutes

erythropoietic activity can maintain normal peripheral values even in the face of hemolysis i e a compensated hemolytic state The hemoglobins and hematocrits in rats injected with the thermostable plasma factor do not show significant change However erythrocytosis is evident Although the latter could conceivably reflect an element of hemoconcentration comparable increases in the hemoglobin and hematocrit levels would then be anticipated in view of the magnitude of the erythrocytosis that these animals manifest Therefore the production of increased numbers of small cells without evidence of augmentation in hemoglobin synthesis rules against hemolysis

The presence of spherocytes would be compatible with both hemolysis and the abnormal Price Jones curves but such cells would possess a normal hemoglobin content even though their diameters were decreased Decreased osmotic resistance should then be demonstrable in recipients of this humoral factor by both the quantitative photocolorimetric and direct cell enumeration techniques Furthermore increased hemoglobin and hematocrit levels should have accompanied the erythrocytosis or else the red blood cell counts should have remained unchanged In addition the erythropoietic stimulation induced by hemolysis is characterized *in vivo* by an increased production of red cells with a normal hemoglobin content Consequently the development of erythrocytoses in the face of stable hemoglobin and hematocrit determinations is incompatible with the concept that the microcytes are normal cells which have assumed the characteristics of spherocytes It then follows that the unique combination of findings observed in normal rats given the thermostable ether soluble factor cannot be due to the action of a hemolytic agent with an effect on red cells such as snake venom lysolecithin or saponin [63-76]

The above conclusion does not rest solely on the demonstration of erythrocytosis in the recipients of *boiled or ether extracts of* active plasmas even though the magnitude and consistency of this finding would appear to establish its significance The failure of these plasma extracts to enhance the incorporation of iron 59 in hemoglobin of recipients given these materials is in itself sufficient evidence to exclude a compensated hemolytic state If the agent in these plasma extracts exerted a hemolytic effect it would

be necessary as a prerequisite for the maintenance of stable hemoglobin and hematocrit values to postulate the presence of definite augmentation in hemoglobin production Although this radioisotopic technique of demonstrating enhanced hemoglobin synthesis is relatively insensitive to minor changes the myeloid erythrocytic hyperplasia and reticulocytosis in these recipient animals are marked and occur promptly There would appear to be little doubt that hemolytic activity of this degree whether compensated or not would be reflected by enhanced iron 59 incorporation in hemoglobin Since the oxygen carrying capacity of the blood is maintained without evidence of enhanced hemoglobin synthesis the microcytes produced in response to this particular stimulus cannot be considered nonfunctional Their individual hemoglobin content is reduced but the total circulating hemoglobin remains normal Therefore the persistent reticulocytosis observed in these recipient rats probably reflects the increased number of cells reaching this stage of development

One other possible effect of the thermostable ether soluble factor deserves comment but it too would seem excludable by experimental data The erythrocytosis without change in the hemoglobin or hematocrit determinations could be explained by fragmentation of circulating red cells [68] The lack of poikilocytosis the size of the microcytes and the uniformity of the small cells giving rise to a double peaked Price Jones curve (Fig 8) are against such an occurrence Moreover such a mechanism could not be correlated with the reticulocytosis and myeloid erythrocytic hyperplasia unless the viability of the fragments resulting from erythrocytic cytoclasis was markedly impaired It would then be possible to reconcile all the peripheral blood and marrow findings However under these conditions enhanced iron 59 incorporation in hemoglobin should of necessity be easily demonstrable

The existence of two humoral factors with different natures and modes of action is in agreement with and apparently explains a number of other observations It is of interest in this regard that heat denatured serum has been found to increase the oxygen consumption of marrow cells *in vitro* but does not exert an effect on heme synthesis The latter was reported to be enhanced by unheated serum [69] In these studies serum heated for thirty minutes

in a boiling water bath increased oxygen consumption as much as the unheated material but contained none of the stimulating effect on heme formation detectable in the original serum

The findings of Gordon and his associates[17] in the isolated hind limbs of rats perfused with active plasmas also necessitate the hypothesis that a humoral factor accelerates erythroblastic cellular division Using this technique for demonstrating the presence of erythropoietic activity in test materials these workers have described increases in the number of nucleated red cells and mitotic figures after infusion with active plasmas for only four hours These observations are clearly incompatible with the concept that the humoral erythropoietic stimulus acts solely by increasing the number of rubriblasts derived from the pluripotential myeloid reticulum cells but does not affect the multiplication of differentiated erythrocytic precursors The average mitotic time of nucleated red blood cells is rather similar in all vertebrates and has been estimated by a number of techniques to be in the order of thirty to sixty minutes In the rat thirty minutes would seem to be a reasonable figure [40] However the generation time of erythrocyte precursors in this species is on the basis of several independent measures probably in the range of two to three days [44, 70] A conservative estimate of the time required for maturation from the rubriblast to the prorubricyte or rubricyte stage would appear to be twenty hours or more There is excellent support for the contention that the humoral erythropoietic factors do not hasten the maturation of myeloid erythrocytic elements Therefore the only conclusion permitted by the short term experiments of Gordon and his co workers is that the perfused plasmas augmented the multiplication of pre existent erythrocytic precursors in the isolated rat hind limbs

The apparent dichotomous nature of the humoral erythropoietic regulatory mechanism is susceptible of further experimental proof In order to test the validity of the hypothesis that a well balanced effect of both plasma factors is needed for the marrow to produce erythrocytes which are normal in size and hemoglobin content the administration of the relatively thermolabile factor alone should enhance hemoglobin synthesis in a recipient but the erythrocytes theoretically should be macrocytic Unfortunately

detailed studies of this type are not yet available Gordon[1, 3, 160] has occasionally observed macrocytosis especially in the recipients of urinary materials Additional experiments are indicated but failure to detect such changes would not necessarily invalidate the thesis that the rate of erythroblastic proliferation is also under humoral regulatory control Certain data suggest that the level of activity attributable to the thermostable ether soluble factor may in fact be determined by the humoral agent which enhances hemoglobin synthesis Other alternative explanations that deserve testing include among others the possibility of selective and preferential utilization by myeloid erythrocytic tissue of a single humoral mitotic stimulant which exerts a governing influence over all hemic precursors (see page 169)

For the reasons given together with the knowledge of the variety of experimental and clinical conditions associated with its enhanced activity it may be concluded that the thermostable ether soluble plasma factor does exert a primary stimulatory effect on erythroblastic cellular division in normal rats thereby governing the number of red cells formed The relatively thermolabile factor on the other hand apparently controls the number of erythrocytic precursors derived from the primitive multipotential myeloid reticulum cells and regulates the amount of hemoglobin produced Their combined physiologic activities appear to determine in addition the size and hemoglobin content of the individual erythrocytes

SUMMARY

Plasma serum urine and certain extracts thereof from a variety of donors with anemic or hypoxic hypoxia are capable of exerting a profound effect on erythropoiesis in a recipient of the same or different species Current data indicate that at least two agents with different chemical physical and physiologic characteristics are responsible for the erythropoietic stimulatory activity contained in these materials

One factor is thermostable soluble in ether and most likely a lipid When administered to normal rats it apparently stimulates homeoplastic cellular division of differentiated marrow erythro

cytic precursors without causing increased diversion of the multipotential marrow reticulum cells into erythrocyte production or demonstrable augmentation in hemoglobin synthesis. This effect on erythropoiesis is manifested in the recipients by myeloid erythrocytic hyperplasia, reticulocytosis and erythrocytosis without associated increases in their hemoglobin or hematocrit levels or enhanced incorporation of iron 59 in hemoglobin. The newly formed erythrocytes are microcytic and possess decreased osmotic resistance. Following discontinuation of the exogenous source of the thermostable plasma factor, normal erythrocytic equilibrium is rapidly re-established. This phenomenon is apparently due to the removal from the peripheral circulation of the microcytes whose survival times are shortened to approximately 15 days and their subsequent replacement with a smaller number of normal cells. The impaired viability of the microcytes is believed to reflect changes in size and or shape occasioned by isolated acceleration of the rate of erythroblastic mitotic activity in the absence of a comparable stimulus to hemoglobin formation rather than a specific property of the erythrocytogenic agent.

The other humoral erythropoietic factor is relatively thermolabile, ether insoluble and probably a mucoprotein or closely associated with such a substance. It enhances the synthesis of hemoglobin most likely by increasing the number of erythrocytic elements derived from the primitive multipotential myeloid reticulum cells. Maturation of erythrocytic precursors and the actual synthesis of the hemoglobin molecule are apparently unaffected by the humoral erythropoietic stimulus and proceed at relatively fixed rates independent of the total output by the marrow and predetermined perhaps by the nutritional and hormonal environment.

It is suggested that these two humoral erythropoietic factors control individually the quantity of hemoglobin and the number of erythrocytes produced. In addition to the regulatory effects on these quantitative aspects of erythropoiesis, their combined activities appear to determine the size and hemoglobin content of each erythrocyte.

Many of the evident discrepancies in experimental observations on the humoral control of erythropoiesis may be due to the pres

ence in active materials of two factors with different properties Whole plasmas and sera or extracts prepared by boiling for less than 10 minutes contain both factors and induce in recipient rats an increase in all parameters that reflect erythropoietic activity Ether extracts or plasma filtrates processed by boiling for 30 or more minutes do not increase iron 59 uptake or the circulating hemoglobin levels but do stimulate erythroblastic cellular division

It is evident that many questions in respect to the humoral erythropoietic factors have yet to be answered Although certain conclusions appear justified and do possess experimental support the speculative nature of theories formulated on the basis of responses induced in recipient animals by substances of unknown composition and purity deserves emphasis The exact modes of action and chemical composition of these humoral substances remain *sub judice* However it may be predicted with reasonable certainty that the intensive investigative activity currently in progress in many laboratories will result in their isolation positive identification and probable synthesis in the near future Further studies designed to elucidate more precisely the physiologic properties of the erythropoietic factors will then be greatly facilitated

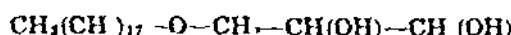
Chapter VI

THE HEMOPOIETIC EFFECTS OF BATYL ALCOHOL

In 1941 *Holmes and his associates* [1] *isolated batyl alcohol* from the nonsaponifiable fraction of bovine yellow bone marrow. This compound (Table IX) had previously been thought to exist

TABLE IX

PHYSICAL AND CHEMICAL CHARACTERISTICS OF BATYL ALCOHOL
(MONOGLYCEROL ETHER OF n OCTADECYL ALCOHOL)



$CH_3(CH_2)_{17}$—O—CH_2—CH(OH)—CH_2(OH)

Molecular Weight 344.56

Melting Point 67.4—70.4 C

White Crystalline Solid

Soluble in the Usual Fat Solvents

only in certain marine organisms. The apparent importance of batyl alcohol as a biologically active substance has been subsequently *emphasized by discoveries of its wide distribution in* nature and its isolation from the spleen [7], arteriosclerotic arteries [3] and erythrocytes, bone marrow, intestinal mucosa, meconium and body cavity fat [4] of a number of mammals including man. Sandler[133] reported in 1949 that batyl alcohol obtained from the yellow bone marrow of cattle stimulated erythropoiesis in normal and benzene treated rats and that an optically inactive synthetic preparation evoked reticulocyte responses in normal human subjects. The similarities between the chemical and physical properties of the *glyceryl ethers and the thermostable ether* soluble plasma erythropoietic factor suggested a possible relation

ship Recent studies in our laboratories on the hemopoietic effects of batyl alcohol have confirmed its erythropoietic stimulatory activity [75]

Six normal female Wistar strain rats were given 20 daily subcutaneous injections of 2 ml of peanut oil containing 12 5 mg of racemic batyl alcohol over a period of four weeks (Saturdays and Sundays excepted) The synthetic batyl alcohol used in this study was extracted with ether from the saline suspension in which it was received the ether evaporated and the crystalline material dissolved in warm peanut oil in a concentration of 6 25 mg of batyl alcohol per ml of peanut oil Another group of animals was given twice the above daily dose for a comparable period of time A third group received peanut oil alone and served as the controls Hemoglobins hematocrits and erythrocyte reticulocyte and marrow nucleated cell counts were determined by the techniques previously described Leukocytes and thrombocytes were enumerated by hemacytometer The latter were counted with the phase microscope using siliconized red cell pipettes and a 1 per cent solution of ammonium oxalate as the diluent

Erythropoietic stimulation was manifested in the animals injected with batyl alcohol by the development of erythrocytosis and reticulocytosis without an associated increase in their hemoglobins or hematocrits (Fig 13) At the end of the injection period the microcytes responsible for the erythrocytosis were demonstrable graphically by Price Jones curves (Fig 14) Decreased erythrocyte osmotic resistance was also evident with the direct cell *enumeration technique* (Fig 14) *These osmotic fragility* abnormalities were not detectable by a quantitative photocolorimetric method At the end of the four week treatment period the femoral marrows of three animals in each group were examined Definite erythrocytic hyperplasia was found in the recipients of the batyl alcohol (Fig 15)

Two weeks after the injections were discontinued all values in the animals not killed for marrow studies had returned to normal baseline levels There was no evidence of altered erythropoietic activity in the control group injected with peanut oil All animals remained healthy gained weight normally and demonstrated no local or general adverse effects from the injections

These findings which have since been reduplicated in a large number of recipients are in agreement with those of Sandler[133] and confirm the erythropoietic stimulatory effect of batyl alcohol

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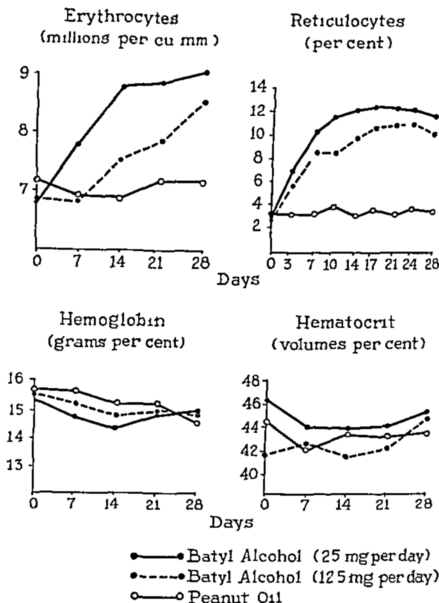


FIGURE 13 Erythrocytosis and reticulocytosis without associated increases in hemoglobin or hematocrit levels in normal rats injected daily for four weeks with a solution of batyl alcohol in peanut oil Average determinations of six animals in each group (From Linman J W Bethell F H and Long M J The erythropoietic stimulatory activity of batyl alcohol *J Lab and Clin Med* 52 596 604 1958 Reprinted by permission)

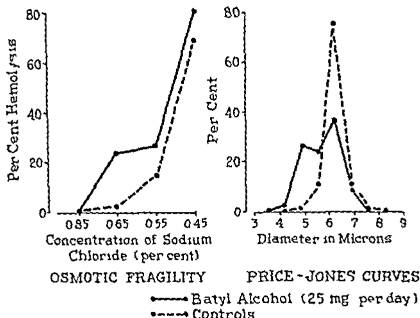


FIGURE 14 Microcytosis and decreased osmotic resistance in recipients of batyl alcohol Composite curves of osmotic fragility determined by a direct cell enumeration technique and red cell diameter distribution (Price Jones) of six rats given 25 mg of batyl alcohol per day and the same number of control animals at the end of a four week injection period (From Linman J W Bethell F H and Long M J The erythropoietic stimulatory activity of batyl alcohol *J Lab and Clin Med* 52 596 604 1958 Reprinted by permission)

in normal rats The greater amounts of batyl alcohol used in our experiments than those employed by Sandler undoubtedly account for the more marked increase in erythrocytes that we observed This apparent relationship between dosage and the degree of response in the recipients was suggested by Sandler s studies and is supported by ours Augmentation in the numbers of erythrocytes and reticulocytes was greater and appeared earlier in the *rats injected with 25 mg of batyl alcohol* per day than in those given half this amount (Fig 13) Erythrocytosis was not evident in the animals given daily injections of 12 5 mg until the end of the second week whereas the rats receiving 25 mg of batyl

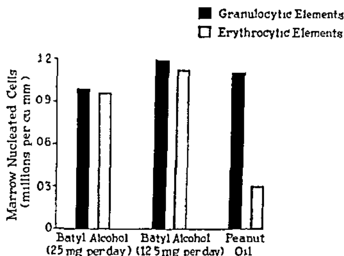


FIGURE 15 Myeloid erythrocytic hyperplasia without a significant increase in marrow granulocytic cell counts in normal rats injected daily for four weeks with batyl alcohol Megakaryocytes were also increased in number Average counts of three animals in each group (From Linman J W Bethell F H and Long M J The erythropoietic stimulatory activity of batyl alcohol *J Lab and Clin Med* 52 596 604 1958 Reprinted by permission)

alcohol per day had an average increase in erythrocytes of approximately 1 000 000 *per cu mm after only five injections* However at the time the injections were discontinued the mean increases in erythrocytes per cu mm over their respective pretreatment values were 1 640 000 in the former group and 2 180 000 in the latter A similar but less definite disparity in the reticulocyte response was also noted (Fig 13)

This singular type of erythrocytic response induced in normal rats by batyl alcohol was identical in all respects demonstrable by the methods used to that which has otherwise been observed only following the administration of boiled or ether extracts of erythropoietically active plasmas (see Chapter V) In addition batyl alcohol does not enhance the incorporation of iron 59 in hemoglobin of recipient animals (Table X) Therefore it may be concluded that batyl alcohol also accelerates the rate of erythro

TABLE X

FAILURE OF BATYL ALCOHOL (25 MG PER DAY x 3) TO ENHANCE THE INCORPORATION OF IRON 59 IN HEMOGLOBIN OF RECIPIENT RATS AVERAGE DETERMINATIONS OF 4 ANIMALS IN EACH GROUP ±1 STANDARD DEVIATION (From Linman J W Long M J Korst D R and Bethell F H Studies on the stimulation of hemopoiesis by batyl alcohol *J Lab and Clin Med* 54 335 343 1959 Reprinted by permission)

Type of Recipients	*Per Cent Iron-59 RBC Uptake at 24 Hours*		
	Normal Serum	*Batyl Alcohol*	*Anemic Serum*
4 Day Starved Rats	6 6±2 7	6 5±1 6	19 9±7 4
14 Day Post hypophysectomized Rats	2 [illegible]±0 5	3 1±2 2	10 9±4 6

blastic proliferation but does not augment the synthesis of hemo globin

The stimulatory effect of batyl alcohol on erythropoiesis might be explained on the basis of 1) Enhanced plasma erythropoietic activity either through increased endogenous production of the humoral factors or antagonistic action on inhibitor substances 2) A stimulatory action on erythropoiesis unrelated to the plasma erythropoietic factors which have been studied 3) Hemolysis or erythrocytic fragmentation rather than primary stimulation 4) Identification of batyl alcohol with the thermostable ether soluble plasma factor or close chemical relationship to it possibly as precursor material

The first hypothesis is susceptible of experimental proof which is not yet available Stimulation of erythropoiesis by anemic or hypoxic hypoxia or by the administration of cobalt ion which probably acts by interfering with oxygen transport has been shown to augment endogenous production of and to be mediated by the humoral erythropoietic factors (see Chapter VII) Similar findings in regard to batyl alcohol would appear unlikely because hypoxia or interference with oxygen metabolism the apparent *sine qua non* for enhanced plasma activity in all other situations

would not be expected to be produced by a compound such as batyl alcohol Moreover if batyl alcohol did affect oxygenation in such a manner as to stimulate erythropoiesis an increase in the factors responsible for both enhanced hemoglobin synthesis and accelerated erythroblastic cellular division would be expected to occur The erythropoietic stimulus imparted by hypoxia in subjects capable of response is always followed by elevation of both hemoglobin and erythrocyte values Consequently it would be difficult to envision as an explanation for the erythropoietic activity of batyl alcohol enhanced elaboration of the thermostable plasma factor alone Nevertheless it is conceivable that batyl alcohol might inactivate an inhibitor of the thermostable factor Additional information is needed before this question can be answered

The theory that batyl alcohol acts independently of the plasma erythropoietic factors is at present merely an explanation by exclusion It is now being subjected to test by assaying for increased erythropoietic activity the plasma of animals made polycythemic by means of batyl alcohol and by attempts to relate chemically batyl alcohol and the erythropoietic stimulating factor in the boiled or ether soluble fractions of active plasmas If both of these experimental approaches should yield negative results some other explanation for the action of batyl alcohol on erythropoiesis not mediated through humoral factors must be sought Since all of the other known erythropoietic stimuli appear to be transmitted to the myeloid reticulum by the humoral factors it would be necessary to ascribe a hitherto unrecognized and highly individualized physiologic effect to batyl alcohol if the above explanation is valid Furthermore it would seem quite unusual for two entirely unrelated biologically active materials i e batyl alcohol and the thermostable ether soluble plasma factor to evoke the same apparently unique type of erythropoietic response in normal recipient animals

The possibility that the erythropoietic response ascribed to batyl alcohol might be secondary to hemolysis rather than true erythropoietic stimulation certainly must be ruled out A number of lipid substances do possess hemolytic properties However the combination of findings in normal rats given batyl alcohol would

appear to exclude hemolysis for the same reasons applicable to the thermostable plasma erythropoietic factor (see page 91). The myeloid erythrocytic hyperplasia and reticulocytosis even in the face of stable hemoglobin and hematocrit values are compatible with a compensated hemolytic state. The erythrocytosis is not. If it reflected erythrocytic fragments with decreased survival times it would again be necessary to postulate definite augmentation in hemoglobin production as a prerequisite for the maintenance of normal hemoglobin and hematocrit levels. Under these circumstances enhanced incorporation of iron 59 in hemoglobin of recipients given batyl alcohol should be evident. Since this is not the case, it would not seem possible to assign hemolytic properties to this compound. Consequently it may be reasonably concluded that batyl alcohol does exert a primary stimulatory effect on erythropoiesis, at least in the normal nonanemic rat.

Evidence currently at hand favors the fourth hypothesis, a close chemical relationship between batyl alcohol and the thermostable ether soluble plasma erythropoietic factor. Both of these substances apparently stimulate homeoplastic mitotic division of erythrocyte precursors without augmenting hemoglobin production; both are resistant to heating to 100° C for prolonged periods; they appear to be of comparable molecular size; batyl alcohol is a monoglycerol and the thermostable factor in plasma also appears to be a lipid.

In addition to its erythropoietic activity, batyl alcohol exerts other effects on hemopoiesis. An increase in the circulating thrombocytes was also observed in the rats injected with this substance. Although the thrombocytosis was not marked, it was definite, bore a relationship to dosage comparable to that noted in respect to the erythrocyte and reticulocyte responses, and has been a consistent finding in all animals given batyl alcohol. The return of the platelet counts to normal baseline and control values one week after the treatment was discontinued adds support to evidence favoring the existence of a specific thrombocytosis promoting property of batyl alcohol.

Significant leukocytosis or myeloid granulocytic hyperplasia (Fig. 15) were not noted in these rats given daily injections of 25 mg. or less, but other studies indicate that this compound does

would not be expected to be produced by a compound such as batyl alcohol. Moreover if batyl alcohol did affect oxygenation in such a manner as to stimulate erythropoiesis an increase in the factors responsible for both enhanced hemoglobin synthesis and accelerated erythroblastic cellular division would be expected to occur. The erythropoietic stimulus imparted by hypoxia in subjects capable of response is always followed by elevation of both hemoglobin and erythrocyte values. Consequently it would be difficult to envision as an explanation for the erythropoietic activity of batyl alcohol enhanced elaboration of the thermostable plasma factor alone. Nevertheless it is conceivable that batyl alcohol might inactivate an inhibitor of the thermostable factor. Additional information is needed before this question can be answered.

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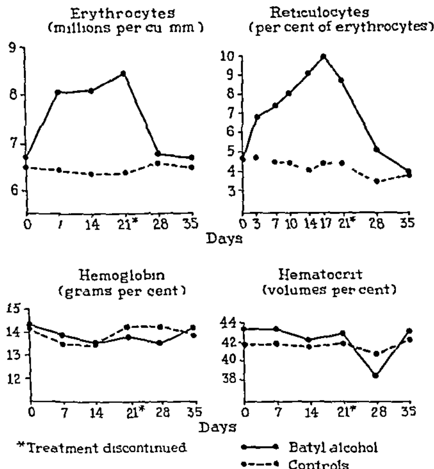


FIGURE 16 Erythrocytosis and reticulocytosis in normal rats given 50 mg of batyl alcohol daily by gastric intubation. This erythrocytic response was identical to those observed following the injection of batyl alcohol (Fig 13) or the thermostable plasma erythropoietic factor (Figs 1 and 7) The explanation for the drop in the hematocrits one week after the treatment was stopped is not apparent Since it was not accompanied by a corresponding decrease in hemoglobin levels and has not been observed in other animals given batyl alcohol it is probably not significant Average determinations of ten animals in each group The controls received comparable amounts of distilled water by the same route (From Linman J W Long M J Korst D R and Bethell F H Studies on the stimulation of hemopoiesis by batyl alcohol *J Lab and Clin Med* 54 335 343 1959 Reprinted by permission)

possess granulocytic stimulatory activity In our early experiments we employed a suspension of batyl alcohol in isotonic saline in doses as high as 75 mg per day With this preparation and dose the recipient animals developed indurated areas at the injection sites which at autopsy were found to contain material grossly similar to that injected The variability of the hematologic changes in these animals was attributed to faulty absorption However the animals that did respond developed leukocytosis in addition to erythrocytosis and thrombocytosis These findings suggested that greater amounts might be required to induce leukocytosis than were needed in order to elicit erythrocyte and thrombocyte increases

The administration of batyl alcohol in peanut oil was not associated with reactions at the injection sites or evidence of impaired absorption but evaluation of large doses with this vehicle was precluded because of difficulties in achieving a concentrated solution Therefore studies were designed to develop and test solutions in other media by oral as well as parenteral routes of administration in the hope that they might answer the question of the granulocytic stimulating activity of batyl alcohol These experiments are still in progress but some observations are available [1, 9]

Twenty normal female Wistar strain rats were divided into groups of ten each One group was given 15 daily 50 mg doses of a suspension in water of batyl alcohol via gastric intubation over a period of three weeks The second group received comparable amounts of distilled water by the same route At the conclusion of the three week period five animals in each group were killed and femoral marrow examined The remaining rats were followed for an additional two weeks to evaluate the rapidity of return to normal hemic equilibrium All animals remained healthy showed no adverse effects from the batyl alcohol and tolerated the daily gastric intubations without untoward reaction The erythroid values leukocytes thrombocytes and marrow counts were determined by the techniques previously described Absolute numbers of the various leukocytic elements in the peripheral blood were derived from the total nucleated cell counts and 200 cell differential counts

This experiment confirms the oral effectiveness of batyl alcohol These recipients also developed erythrocytosis and reticulocytosis without associated increases in their hemoglobin or hematocrit levels (Fig 16) In addition they manifested thrombocytosis and leukocytosis (Fig 17) The latter was due chiefly to an in

TABLE XI

CIRCULATING NUCLEATED CELLS PER CU MM IN NORMAL RATS GIVEN BATYL ALCOHOL ORALLY (50 MG PER DAY × 15) THE CONTROLS RECEIVED COMPARABLE AMOUNTS OF DISTILLED WATER AVERAGE VALUES OF 10 RATS IN EACH GROUP (From Linman J W Long M J Korst D R and Bethell F H Studies on the stimulation of hemopoiesis by batyl alcohol *J Lab and Clin Med* 54 335-343 1959 Reprinted by permission)

Cell Type	*Batyl Alcohol*		*Controls*	
	Baseline	*3 Weeks*	*Baseline*	*3 Weeks*
Total Leukocytes	11 800	19 100	13 800	12 900
Neutrophils	1 128	5 2[illegible]2	1 704	1 80[illegible]
Lymphocytes	10 167	13 033	11 5[illegible]	10 491
Monocytes	323	598	388	283
Eosinophils	160	401	9[illegible]	137
Basophils	27	26	53	23

rapid return to normal after the treatment was stopped are such that a causal relationship cannot be denied The myeloid hyperplasia and stable hemoglobin and hematocrit levels eliminate hemoconcentration as a cause for the findings Consequently primary myelopoietic stimulation or hemolysis would appear to be the only alternative explanations for the hematologic phenomena observed Although thrombocytosis and leukocytosis do accompany hemolysis the evidence previously cited (see page 105) appears to exclude a hemolytic effect

Although the exact significance of the hemopoietic effects of batyl alcohol must await future experimentation these data afford strong support for the conclusion that this compound stimulates the proliferation of myeloid elements The necessity of giving relatively large doses is in all probability the explanation for the negative and equivocal results that have been noted Evenstein Gordon and Eisler [78] have given synthetic batyl al

crease in granulocytic elements (Table XI) The changes in the peripheral blood were accompanied by increased numbers of both nucleated erythrocytic and granulocytic cells in the marrow (Fig 18) and megakaryocytes were very numerous All

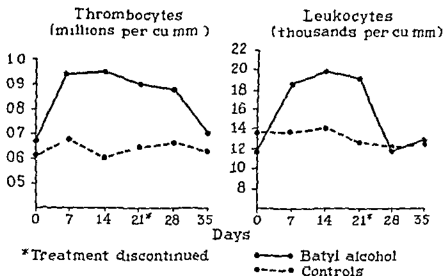


FIGURE 17 Thrombocytosis and leukocytosis in normal rats given 50 mg of batyl alcohol daily by gastric intubation The increase in leukocytes was *chiefly due to a relative and absolute neutrophilia* The thrombocyte and leukocyte counts had returned to normal two weeks after the treatment was stopped Average counts of ten animals in each group (From Linman J W Long M J Korst D R and Bethell F H Studies on the stimulation of hemopoiesis by batyl alcohol *J Lab and Clin Med* *54* 335 343 1959 Reprinted by permission)

determinations in the animals not killed for marrow studies returned promptly to normal baseline and control levels after the treatment was discontinued These findings indicate that batyl alcohol is capable of stimulating granulopoiesis in normal rats but greater amounts are apparently required to elicit this response than are needed to induce erythrocytosis or thrombocytosis

The magnitude and uniformity of the erythrocyte reticulocyte thrombocyte and leukocyte increases in the recipients of batyl alcohol (Table XII) together with the marrow findings and the

TABLE VII

STANDARD DEVIATIONS IN THE HEMATOLOGIC VALUES OF 10 RATS GIVEN 50 MG OF BATYL ALCOHOL VIA GASTRIC INTUBATION DAILY AND OF 10 RATS GIVEN DISTILLED WATER BY THE SAME ROUTE (From Linman J W Long M J Korst D R and Bethell F H Studies on the stimulation of hemopoiesis by batyl alcohol *J Lab and Clin Med* 54 335 343 1959 Reprinted by permission)

Determination	*Groups*	*Baseline*		*3 Weeks**		*5 Weeks*	
		Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*
RBC S x 10⁶	Batyl Alcohol	6 68	0 48	8 42	0 44	6 66	0 43
	Controls	6 49	0 33	6 39	0 30	5 91	0 57
Platelets x 10⁶	Batyl Alcohol	677	091	896	090	702	041
	Controls	608	111	646	099	624	066
WBC S x 10³	Batyl Alcohol	11 8	1 4	19 1	2 7	12 9	2 2
	Controls	13 8	2 2	12 9	2 7	12 6	3 2
Retics %	Batyl Alcohol	4 6	1 5	8 7	1 1	3 9	0 2
	Controls	4 6	2 0	4 3	0 7	3 7	0 4

*Treatment discontinued

The concept of a biologically active substance capable of altering the rate of cellular proliferation is not without precedent A number of agents which promote cell division in plants have been described [77] The possibility that a lipid may play a basic role in the control of hemopoiesis is also supported by several experimental observations Evans and co workers [33, 34] have shown that hemorrhage or the administration of phenylhydrazine are followed by the preferential removal of linoleic and linolenic acids from the marrow fat of rabbits Even though the eventual fate of

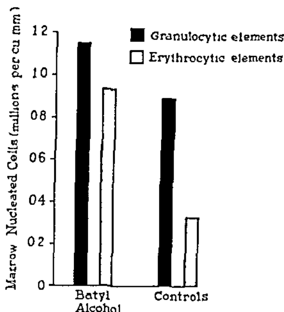


FIGURE 18 Average marrow nucleated cell counts of five rats in each group demonstrating both erythrocytic and granulocytic hyperplasia in the recipients of 50 mg of batyl alcohol orally per day for three weeks (From Linman J W Long M J Korst D R and Bethell F H Studies on the stimulation of hemopoiesis by batyl alcohol *J Lab and Clin Med* 54 335 343 1959 Reprinted by permission)

cohol in doses of one to five mg daily to rats No alterations in hemopoiesis were noted over periods of one to four weeks We have also failed to obtain responses with such small amounts

The demonstration of the thrombopoietic and granulopoietic activity of batyl alcohol in addition to its effect on erythropoiesis bears directly on the problem of the control of hemopoiesis These observations are in apparent accord with the hypothesis that all aspects of myelopoiesis may be under humoral regulatory control and that a single substance or activator inhibitor complex may affect the formation of all hemic elements derived from the pluri potential myeloid reticulum cells Batyl alcohol originating in yellow bone marrow may be of primary importance in such a system

been isolated from yellow bone marrow 2) This glyceryl ether appears to stimulate cellular proliferation of myeloid elements in the intact rat 3) Thrombocytic and erythrocytic precursors are apparently more sensitive to the stimulus imparted by batyl alcohol than are cells of the granulocytic series Tentative conclusions are neither justified nor implied and further speculation would at this time be hazardous

Many questions remain unanswered as to what role if any batyl alcohol may play in the physiologic or pathophysiologic control of hemopoiesis Foremost among these is the apparent inconsistency between the dosage of batyl alcohol required to alter myelopoiesis in normal rats and the much smaller amounts of a substance possessing such specific biologic activity that would be expected to exert a readily detectable effect Conceivable explanations for this observation include the difficulties with absorption previously mentioned with exogenous requirements greatly exceeding endogenous needs the use of a racemic synthetic preparation instead of the optically active natural form and the possibility that batyl alcohol may be closely related to or a precursor of but not identical with some other more active substance Additional studies are now in progress in our laboratories and those of other investigators pertaining to the hemopoietic activities of the other glyceryl ethers selachyl and chimyl alcohol Although the latter is also a solid selachyl alcohol which differs only slightly from batyl alcohol in chemical structure is liquid at room temperature and is the glyceryl ether found most abundantly in nature [80]

The possible therapeutic potentialities of a substance such as batyl alcohol are readily apparent and deserve exploration A few observations of this type are available It has been reported that batyl alcohol exerts a therapeutic effect in mice and patients with post irradiation leukopenia [81-83] Evans and co workers [84] have also described beneficial results following the administration of batyl alcohol to cattle with Bracken poisoning a disorder associated with pancytopenia due to ingestion of the Bracken fern However Schultze and his associates [85] were unable to prevent or alter with batyl alcohol the course of aplastic anemia induced in calves by the administration of trichloroethylene extracted soybean oil meal The site type and degree of injury to the myeloid

these fatty acids is unknown it may be reasonably surmised that they take part in the erythropoietic response to such stimuli In this regard it is interesting that these workers [34] were unable to relate the loss of these fatty acids from the marrow to the synthesis of hemoglobin Batyl alcohol also fails to enhance hemoglobin production but still exerts a clear cut effect on hemopoiesis

In addition to the loss of lipid material from marrows under going active regeneration hemorrhage is known to be associated with an increase in blood lipids It has also been shown that the application of a variety of hypoxic stimuli is quickly followed by an increase in certain plasma esterases [78] thus suggesting the operation of a lipolytic enzyme system in the ensuing erythropoietic response

Steinberg [79] has reported studies which indicate that myelopoiesis is predicated on the presence of fat cells in the marrow In these well conceived experiments marrow was extirpated from one or more of the long bones of normal living rabbits and the pattern of regeneration was studied The formation of fat cells consistently followed the sprouting of primitive reticulum cells and always preceded the appearance of differentiated hemic elements When the rabbits were given large amounts of benzene regeneration did not progress further than the appearance of a few primitive reticulum cells With lesser degrees of intoxication however *this stage was followed by the formation of fat cells and* then by the proliferation of hemic precursors An interesting sequential pattern of regeneration was observed Megakaryocytes appeared first with the subsequent development of foci of erythrocytic precursors Granulocytes were not noted unless a considerable number of fat cells developed and not until megakaryocytes and cells of the erythrocytic series were present in moderate numbers Steinberg concluded that myeloid activity is dependent on fat cells and that at least part of the mechanism by which benzene induces marrow aplasia is by inhibiting cellular division

In view of the meager experimental data currently available it is obviously impossible to relate in any way the above findings to the hemopoietic effects of batyl alcohol in normal rats Even so the following points are worthy of note 1) Batyl alcohol has

been isolated from yellow bone marrow. 2) This glyceryl ether appears to stimulate cellular proliferation of myeloid elements in the intact rat. 3) Thrombocytic and erythrocytic precursors are apparently more sensitive to the stimulus imparted by batyl alcohol than are cells of the granulocytic series. Tentative conclusions are neither justified nor implied, and further speculation would at this time be hazardous.

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elements are undoubtedly of paramount importance in determining the ultimate response Widespread and irreversible damage to the myeloid reticulum could prevent a beneficial effect regardless of the potency of the stimulus imparted by the batyl alcohol It is not possible to relate these observations to ours since we utilized normal animals as recipients Yet it is of interest that on the basis of weight alone the smallest daily dose which we have found to be effective in normal rats is approximately eight times that used by Schultze and his co investigators

Since batyl alcohol has been isolated from yellow bone marrow it seems probable that the therapeutic results attributed in the past to yellow bone marrow and extracts thereof [46, 47] were due to its presence Recovery in benzene poisoned rabbits given the unsaponifiable fractions of yellow bone marrow has also been described [48] Observations on the myelopoietic effect of batyl alcohol support the once widely held concept that yellow bone marrow extracts and concentrates do possess hemopoietic stimulatory properties *Insufficient dosage may be the reason they* fell into disrepute as therapeutic agents

The chemical and physiologic attributes common to batyl alcohol and the ether soluble thermostable plasma erythropoietic factor suggest reasonably but without proof that they are closely related If this relationship is established by future investigation the pathogenesis of the thrombocyte and leukocyte increases associated with acute hemorrhage some types of hemolysis and polycythemia vera may be clarified The failure to observe consistently leukocytosis and thrombocytosis in experimental animals or human subjects with hyperactive erythropoiesis due to endogenously enhanced humoral factor activity does not necessarily rule against this possibility It may reflect varying degrees of sensitivity of the different myeloid elements comparable to the dose response relationship noted in normal rats given batyl alcohol In this connection there does exist a suggestive correlation between thrombocytosis leukocytosis and the severity of certain acute hemolytic processes Moreover the granulocyte and thrombocyte increases in acute hemorrhage are related to the magnitude and rapidity of the blood loss and disappear after subsidence of the initial and presumably maximal hypoxic stimulus

The reported failures of plasma from anemic or hypoxic donors to induce leukocytosis in recipient animals[119, 1, 1, 1, 3, 1, 6, 19, 9] may be similarly explained. Observations on the granulopoietic activity of larger doses of "anemic" plasmas are needed as are those on the thrombocytosis promoting properties of such materials. Support for the thesis that the thermostable plasma erythropoietic factor may also affect the rate of proliferation of other hemic precursors has recently been obtained in patients with polycythemia vera (see page 161). It should be emphasized, however, that this suggested relationship between batyl alcohol and the thermostable plasma factor is as yet conjectural and without incontrovertible experimental documentation.

In summary, our preliminary studies have shown that batyl alcohol, the monoglycerol ether of n-octadecyl alcohol, induces the same unique type of erythropoietic response in normal rats as that observed following the administration of the thermostable or ether soluble fractions of erythropoietically active plasmas. It is suggested, although not yet proved, that batyl alcohol may be identical with or closely related to this plasma erythropoietic factor, perhaps as precursor material. Batyl alcohol also evokes thrombocytosis and granulocytosis in normal rats, thereby lending support to the thesis that all aspects of myelopoiesis may be subject to humoral regulatory control. These findings assume added interest in the light of recent advances in our knowledge of the mechanisms governing erythropoiesis and may provide a clue to the intriguing mystery that surrounds the homeostatic control of hemopoiesis.

Chapter VII

CONTROL OF PLASMA ERYTHROPOIETIC ACTIVITY

Enhanced plasma erythropoietic activity has been found in a number of diverse clinical and experimental conditions However all of these situations with the single exception of polycythemia vera have some type of hypoxia* as a common denominator This finding in itself strongly suggests that there is a causal relationship between hypoxia and the level of erythropoietic factor activity Many experimental observations support and confirm this hypothesis

The erythropoietic stimulatory effect of hypoxia was recognized many years before a humoral regulatory mechanism was first postulated Hypoxia is now generally accepted as the fundamental stimulus for erythropoiesis Prior to the demonstration of plasma factors which are capable of accelerating erythropoiesis in normal recipient animals the production of blood loss anemia exposure to lowered barometric pressure or the administration of cobalt constituted the only means of experimentally inducing erythropoietic stimulation It is essential to consider the evidence favoring oxygen deficiency as the determinant of plasma factor activity before the latter can be assigned a basic role in the primary control of erythropoiesis

The erythropoietic stimulus imparted by hypoxic hypoxia irrespective of the mechanism responsible for the reduction in arterial oxygen saturation has been extensively studied and is an indisputable fact This erythropoietic response gave rise to the theory that hypoxia of bone marrow elements was the fundamental stimulus to erythropoiesis It was subsequently inferred that the

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* The term hypoxia is used throughout this discussion to designate cellular oxygen deficiency Four types are recognized 1) Anemic hypoxia (diminution in the oxygen carrying capacity of the blood) 2) hypoxic hypoxia (reduction in the arterial oxygen saturation or pO_2) 3) histotoxic hypoxia (impaired utilization of oxygen by the tissues) and 4) stagnant hypoxia (circulatory insufficiency)

increased erythropoietic activity seen in certain types of anemia was due to a decrease in the oxygen-carrying capacity of the blood and the concept of marrow hypoxia as the primary erythropoietic stimulant was extended to include anemic hypoxia. This thesis was understandable and even desirable in an attempt to establish a single explanation for the control of erythropoiesis. However, it was based on indirect evidence and was developed without knowledge of the oxygen content of the marrow. Although the role of hypoxia in the regulation of red cell production cannot be denied, the theory in regard to a direct marrow effect has since been disproved (see page 20). As a result it became necessary to explain by other means the erythropoietic stimulation induced by hypoxic or anemic hypoxia. Mediation of the hypoxic stimulus by a humoral factor(s) offered a logical explanation and no longer lacks experimental support.

The presence of humoral erythropoietic factors in animals exposed to low oxygen tension was first described by Forster in 1924[113] and has since been confirmed by many investigators.[116, 117, illegible] There also exists indirect evidence that hypoxic hypoxia exerts its erythropoietic stimulatory effect via the plasma factors. Examples of such support are found in the experiments of Reissmann[118] with parabiotic rats and those of Grant[illegible] with lactating rats and mice. In the former, erythropoietic stimulation was evident in both partners even though only one animal was maintained at simulated high altitude. In the latter studies, hypoxia of lactating mothers induced accelerated erythropoiesis in their normal offspring. In addition, observations on patients with regional hypoxia[illegible] have implicated a humoral mechanism in the erythropoietic stimulation accompanying such disorders. These patients had localized hypoxia but generalized myeloid erythrocytic hyperplasia. Conversely, increased erythropoiesis is not evident in isolated ischemic limbs.[illegible] Enhanced plasma erythropoietic activity has also been demonstrated in patients with decreased arterial oxygen saturation and secondary polycythemia of varied etiology[149, illegible] and in human subjects residing at high altitudes.[illegible] Therefore it may be logically concluded that the stimulus to erythropoiesis occasioned

by hypoxic hypoxia is mediated through the plasma erythropoietic factors

A relationship between these factors and the hyperactive erythropoiesis associated with hemorrhagic or hemolytic anemia is also readily apparent Carnot and Deflandre[101, 107] were actually studying the mechanisms of regeneration of organs when they noted in the serum of rabbits with active regeneration of blood after bleeding a substance that would stimulate red cell production in other normal rabbits They reported that the potency of the serum was greatest on the first day following bleeding and progressively decreased with repeated bleedings However Forster and Kiss[300] subsequently demonstrated activity in the serum of animals with chronic hemorrhagic anemia The increase in plasma erythropoietic activity in both acute and chronic hemorrhagic anemia has since been confirmed repeatedly

Erslev[08] has described studies concerning the erythropoietic stimulatory effect contained in rabbit plasma after time-limited *exposure to anemic hypoxia He found that the plasma of rabbits* whose hemoglobin had been reduced approximately 50 per cent by a single bleeding was questionably active three hours later Anemia maintained for six twenty and forty eight hours progressively induced greater plasma factor activity The activity present after five days was similar to that observed twenty and forty eight hours after bleeding The correction of anemia of twenty hours duration reduced the plasma activity to undetectable levels in three hours Erslev also reported similar observations following time limited exposure to simulated high altitudes It is evident from these experiments and others that both anemic and hypoxic hypoxia evoke a prompt increase in plasma erythropoietic factor activity

Gibelli[110] *first described erythropoietic properties in the serum* of animals with acute phenylhydrazine induced hemolytic anemia This technique has received widespread use as a method of *augmenting plasma activity* because of its reliability reproducibility and relative simplicity Consequently it is apparent that the lowered erythroid values brought about by hemorrhage or hemolysis enhance the endogenous plasma erythropoietic activity The humoral factors are undoubtedly responsible for the

in vivo increase in erythropoiesis demonstrable under these conditions

Even though the arterial and bone marrow oxygen saturation in hemorrhagic and hemolytic anemias are normal the oxygen carrying capacity of the blood is reduced and anemic hypoxia results Animals exposed to lowered barometric pressures or rendered anemic by hemorrhage or the administration of phenyl hydrazine have three things in common 1) increased plasma erythropoietic activity 2) myeloid erythrocytic hyperplasia and 3) hypoxia Since the accelerated erythropoiesis is most likely the result of the increased plasma activity it then follows that hypoxia whether hypoxic or anemic in type must be the stimulus to the augmentation of the humoral erythropoietic factors The remote possibility that the latter might represent a by product of hyperactive myeloid erythrocytic elements can be excluded on the basis of observations on the effects of nitrogen mustard [11] and irradiation[1 7] on plasma factor activity (see page 136) These studies have shown that a regenerative marrow is not a prerequisite for the formation of the humoral erythropoietic factors

Cobalt is another means of evoking accelerated erythropoiesis in many species [115] This response is manifested by a true polycythemia with an increase in circulating hemoglobin and red cell mass erythrocytosis reticulocytosis and myeloid erythrocytic hyperplasia Large doses eventually depress red cell production [301] The mechanism by which cobalt exerts such an effect on erythropoiesis has been clarified by recent observations The cobaltous ion is known to have an inhibitory effect on the endogenous respiration of a number of tissues There exists evidence which indicates that it interferes with certain enzymes concerned with the transport and utilization of oxygen including succinic dehydrogenase choline oxidase cytochrome oxidase and catalase [30] Such an effect would result in histiotoxic hypoxia Since hypoxia of marrow elements does not constitute a specific stimulus to erythropoiesis and because cobalt does not stimulate either oxygen consumption or heme synthesis in bone marrow cultures *in vitro* [303] it is necessary to postulate some mechanism other than a direct effect on erythrocytic tissue Augmentation in plasma erythropoietic activity is known to follow hypoxia and has been

described in animals given cobalt[304-306] Therefore it would appear that the erythropoietic stimulatory effect of cobalt is also mediated through the plasma factors and most likely dependent on the production of tissue hypoxia The possibility that cobalt itself might be responsible for the erythropoietic activity in the plasma of animals treated with this agent can be excluded A very small amount of cobaltous ion is present in such plasmas whereas relatively large quantities of the metal must be administered to produce polycythemia

An increase in plasma erythropoietic activity derived from either exogenous or endogenous sources is the apparent *sine qua non* for the stimulation of erythropoiesis in the normal experimental animal Since all methods of enhancing humoral activity are associated with hypoxia it may be inferred with reasonable certainty that general or local tissue hypoxia is the stimulus to the elaboration or activation of the plasma factors Erslev[307] has shown that dilution anemia secondary to the administration of dextran does not cause increased erythropoiesis despite the reduced oxygen concentration of arterial blood This finding indicates that local tissue oxygen tension is probably the method by which the level of plasma stimulatory activity is controlled Pending further study it must be assumed that the functional level of each plasma factor is dependent upon hypoxia It is entirely possible however that the manner by which hypoxia exerts this regulatory effect on the humoral erythrocytogenic agents may differ It may for example stimulate or control the formation release or activation of only one factor which is then responsible for exerting a similar effect on the other

In addition to the enhanced plasma erythropoietic activity induced experimentally by hemorrhage phenylhydrazine hypoxic hypoxia and cobalt plasmas from fetal sheep[2 8] and dogs [04] normal pregnant rats[308] and human subjects with secondary polycythemia and a variety of primary and secondary anemias have been shown to be active Umbilical cord blood of the normal newborn[0 03 05 31 309] and plasma from patients with congestive heart failure[117] have also been reported to stimulate erythropoiesis in recipient animals All of these conditions are associated with some type of hypoxia thereby strengthening the theory that tissue

hypoxia controls the functional level of the humoral erythropoietic factors The only exception to the correlation between hypoxia and increased plasma erythropoietic activity is found in patients with polycythemia vera However this does not detract from the importance of hypoxia as a determinant of humoral factor activity There is good reason to believe that an idiopathic over production of these humoral agents is of etiologic significance in polycythemia vera (see page 157)

On the basis of numerous experimental and clinical observations it may be concluded that hypoxia is the fundamental or basic stimulus to erythropoiesis and that the humoral factors constitute the mechanism by which this stimulus is mediated to the myeloid reticulum Such a unitarian concept of the control of erythropoiesis is obviously desirable but in a living organism where blood and oxygen supply to various tissues are under continuous flux the possibility that other regulatory mechanisms may be operating must not be overlooked

Stohlman [5] has recently called attention to several observations which argue against the thesis that hypoxia alone governs the rate of red cell production These include a number of situations associated with enhanced erythropoiesis without demonstrable hypoxia e g the compensated hemolytic states frequently seen in patients with hereditary spherocytosis and in dogs following the insertion of a lucite ball prosthesis into the cardiovascular system [310, 311] Furthermore the loss of very small amounts of blood would hardly seem capable of altering the oxygen carrying capacity of the blood more than the daily fluctuations in blood flow that occur normally Yet it has been shown that the peripheral erythroid values remain stable in normal human subjects bled 20 to 30 ml every other day for three months [31] Other evidence includes the potentiating effect of cobalt on the erythropoietic response to hemorrhage[313] or lowered barometric pressure [314] Altland and Parker[315] have also shown that hypoxic hypoxia severe enough to cause death in the box turtle failed to induce increased erythropoiesis but repeated bleedings did evoke an erythropoietic response Moreover hyperoxia decreases but does not abolish erythropoietic activity in experimental animals and human subjects

In an attempt to explain these findings and the day to day regulation of normal erythrocytic equilibrium Brecher and Stohlman[31] have proposed a dual mechanism of erythropoietic control They have postulated the development of an erythropoietic inhibitor within the red cell during the aging process When the cell is destroyed the inhibitor is released and acts directly on the bone marrow Minimal hemorrhage by decreasing the number of older cells would reduce the amount of this hypothetical inhibitor substance released per day Hemolysis would remove cells prior to the acquisition of the inhibitor It is suggested reasonably but without experimental verification that this mechanism exerts a fine regulatory control over erythropoiesis and that hypoxia through its enhancing effect on humoral erythropoietic factor activity serves as a booster in the event of greater need

Although the data cited admittedly persuade against a unified concept of erythropoietic control several points must be clarified before a definitive conclusion can be reached Alternative explanations may exist Thus the normal erythroid values in compensated hemolytic syndromes may actually be lower than those required for the particular level of metabolic activity present In addition the site at which hypoxia exerts its enhancing effect on the humoral factors is not known It is difficult to visualize just how sensitive it might be to immeasurable changes in tissue oxygen tension The varying responses and degrees of plasma activity noted after the application of different hypoxic stimuli may also be related to such things as the magnitude and type of hypoxia the presence or absence of certain cardiovascular or pulmonary compensatory measures and the depressive effect of marked bone marrow hypoxia on erythropoiesis In regard to the failure of the box turtle to respond to hypoxic hypoxia, it may reflect the vastly different oxygen requirements and much higher carbon dioxide capacity of the blood of poikilotherms as compared to homiotherms

The failure of hyperoxia to abolish erythropoiesis even in the face of a marked increase in alveolar arterial and tissue pO_2 is difficult to reconcile with the hypoxic theory However normal plasma contains small amounts of the plasma factors in the absence of demonstrable hypoxia (page 127) It is possible therefore

that a tissue oxygen tension greater than that which can be achieved by increasing the oxygen content of inspired air may be needed to completely suppress humoral factor activity Although the existence of a second erythropoietic regulatory mechanism cannot be excluded there is as yet no compelling reason to alter the thesis that hypoxia and the plasma erythropoietic factors exert primary regulatory control over red cell production

Hypoxia is undoubtedly the chief determinant of enhanced plasma erythropoietic activity but the assumption that it stimulates the actual formation of one or both of the humoral factors is not warranted It is theoretically possible that hypoxia may alter existent activator inhibitor complexes or bring about the release of preformed factors stored in their formative tissue or elsewhere It is also feasible that the plasma factors may be elaborated as inactive precursors and activated in some manner by hypoxia Gordon[1,3] has postulated that the humoral factors may be formed by a mechanism analogous to that of hypertensin This scheme envisions hypoxia as bringing about the production and release into the circulation of a proteolytic enzyme complex which acts on protein substrate furnished by the blood forming organs to produce the active agent An alternative scheme involves the operation of a lipolytic enzyme system acting on fatty substrate furnished by the marrow These theories are supported by the prompt increases in peptidase and esterase activity in the plasma following hemorrhage exposure to simulated high altitude or the administration of cobalt [79,318]

The level of plasma erythropoietic activity bears a direct relationship to the degree of hypoxia but is also determined in part by the functional status of the marrow Utilization of the active agents by the marrow is undoubtedly the explanation for the higher levels of plasma activity present in human subjects or experimental animals with myeloid hypoplasia than in those with erythrocytic hyperplasia Excretion by the kidneys constitutes *another* potential removal mechanism and the apparent relationship between the level of plasma activity and that in the urine suggests the presence of a threshold for the factors The liver has also been implicated as playing a role in the excretion or inactivation of the humoral erythropoietic substances

The less marked stimulatory capacity of plasma from animals subjected to repeated bleedings as opposed to those with phenylhydrazine induced anemia has been noted by several investigators This observation has usually been explained on the basis of the rapid development and more severe depression in erythroid values in animals given phenylhydrazine However Jacobsen and her associates[186] have reported a correlation between the level of erythropoietic activity and liver damage They noted less plasma activity in bled animals than in those given phenylhydrazine even though the anemia was comparable in both groups These workers also described differences in responses in recipients of phenylhydrazine induced anemic plasma which appeared to be dependent on the extent of liver damage rather than the severity of the anemia All of their phenylhydrazine anemic plasmas were capable of inducing reticulocytosis in recipient animals but only those from donors with liver damage evoked a definite increase in red cell mass as determined by the iron 59 technique It was concluded from these observations that the liver probably participates in the humoral regulatory mechanism by inactivating the active factor

Prentice and Mirand[317] have also postulated that the liver plays a significant role in determining the level of plasma erythropoietic activity These investigators were unable to demonstrate enhanced activity in the plasma of rats subjected for forty eight hours to a low oxygen atmosphere unless prior acute liver damage had been produced by carbon tetrachloride Activity was evident in normal rats after exposure for twenty four hours In spite of these findings they did not observe any greater erythropoietic response in animals with liver damage following such stimuli as hypoxia or the administration of active phenylhydrazine anemic plasma than in the normal controls Observations by a number of other workers have failed to support this concept that liver damage is a prerequisite for augmentation in plasma erythropoietic activity by hypoxic hypoxia It would appear more likely that the relationship noted by Prentice and Mirand was due to the fact that animals maintained at low oxygen tension develop maximal plasma stimulatory activity in approximately twenty four hours followed by the restoration of equilibrium and a rapid decline to

undetectable levels after forty eight to ninety hours The increased activity in their rats given carbon tetrachloride may have reflected a damaged marrow incapable of utilizing the humoral factors i e a situation analogous to Stohlman s experiments with x irradiation [6]

The possible role of the liver in the humoral regulation of erythropoiesis must await investigative clarification but several points deserve comment Although greater plasma activity has been noted in animals with phenylhydrazine induced anemia than in those with anemia secondary to acute or chronic blood loss this difference has not always been marked and in many instances quite minimal The efficacy of hemorrhage as a means of enhancing erythropoietic stimulatory activity cannot be questioned In addition many other conditions are associated with increased plasma activity without definite liver damage Examples include patients with certain types of anemia secondary polycythemia and polycythemia vera If the liver exerted an important inhibitory effect on the plasma factors thereby explaining the greater activity in phenylhydrazine treated donors with liver damage it would be difficult to reconcile the easily demonstrable erythropoietic activity in the absence of liver damage

The reported association between liver damage and the level of activity in donors given phenylhydrazine may be related to individual responses to the drug There is a direct relationship between dosage and the toxic effects of phenylhydrazine which include hemolysis and hepatic damage However as is the case with similar agents all animals will not respond in a like manner to a given dose Variability in the degree of liver damage is expected The tremendous functional reserve of the bone marrow does not permit a strict correlation between the severity of the hemolytic process and the peripheral erythroid values Individual differences in response to phenylhydrazine may be present even though the anemia is comparable in degree It is reasonable then to surmise that liver damage may reflect a more marked reaction to the phenylhydrazine including a greater hemolytic *component In addition the actual decrease in the* oxygen carrying capacity of the blood in recipients of phenylhydrazine is greater than indicated by the degree of anemia because of the

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scribed in recipients of normal human [6] and rabbit plasma [1,6] Gordon and his associates [10] have also reported erythropoietic stimulation in the isolated hind limbs of hypophysectomized rats following perfusion with normal plasma

It has been postulated that the failure to demonstrate activity in normal plasma or plasma extracts (Figs 19 and 20) may be

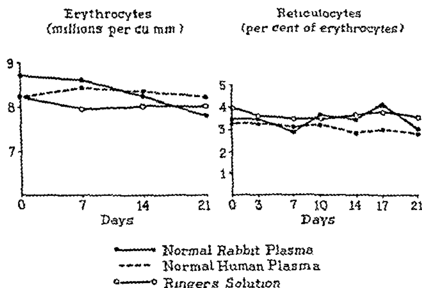


FIGURE 19 Failure of the boiled filtrates of normal rabbit and human plasma to alter the erythrocyte or reticulocyte counts in normal rats over a three week injection period Daily doses were equivalent to 2 per cent of the recipients body weights The hemoglobins and hematocrits also remained stable Average counts of sixteen animals receiving each of the above described materials

due to the low concentration of the factors in such materials and the relative insensitivity of the assay methods Recent studies indicate that this is probably true We have observed definite erythropoietic stimulation in rats given a six to ten times concentrated dose of normal plasma [149] and Gurney and his associates [200] have also detected erythropoietic activity in normal human plasmas concentrated tenfold Although some dissenting opinions

relatively large amounts of methemoglobin present Therefore it is not possible to ascribe a primary role in the humoral regulation of erythropoiesis to the liver The possibility that the liver may exert secondary or modifying effects cannot of course be excluded

It has become increasingly apparent as more data on the plasma factors are accumulated that the mechanisms which control erythropoietic activity are in all probability quite complex At this time the only justifiable conclusion is that hypoxia is an essential and basic determinant of the level of plasma factor activity The manner by which this is accomplished is as yet purely speculative The existence of inactive precursors or activator inhibitor complexes would seem quite likely Furthermore additional information as to the utilization excretion or inactivation of the erythropoietic factors is needed since they may play important roles in the regulation of plasma activity It also should be emphasized that deficiencies of certain substances such as iron and vitamin B_1 exposure to various chemical and physical agents and the presence of pathologic processes which injure or destroy normal myeloid elements are capable of drastically altering or modifying erythropoiesis irrespective of the integrity of the humoral regulatory mechanism

It is evident that the plasma erythropoietic factors exert important regulatory control over erythropoiesis in a number of situations involving either a reduction in the oxygen carrying capacity of the blood or a decrease in arterial oxygen saturation The marked effect of the plasma factors on erythropoiesis under these circumstances certainly suggests that they are also involved in the homeostatic control of the normal erythroid steady state The facts that transfusion polycythemia[83] and high tensions of inspired oxygen[84, 8] depress erythrocytogenesis provide additional support for the role of the humoral factors in normal erythropoiesis

The concept of a humoral erythropoietic regulatory mechanism demands that the responsible factors be demonstrated in the blood of normal as well as hypoxic donors Although most investigators have failed to detect the presence of erythropoietic factors in normal plasmas enhanced iron 59 uptake has been de

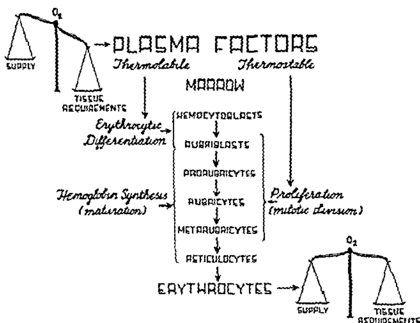


FIGURE 21 Role of the plasma erythropoietic factors in the fundamental control of erythropoiesis

rubriblasts through normal growth to differentiate into mature hemoglobin containing erythrocytes These two aspects of erythropoiesis are probably inseparable and provided with a normal nutritional and hormonal environment appear to proceed at a fixed and predetermined rate Even though the generation time required for the maturation of erythrocytic precursors and the synchronous production of hemoglobin may be unalterable the end result of this process in terms of its contribution to the circulating hemoglobin and red cell mass is subject to both quantitative and qualitative modifications

Little is known concerning the mechanisms which govern the release of erythrocytes from the marrow Under conditions of increased need the premature delivery to the circulation of increased numbers of reticulocytes and other young erythrocytes occurs While capable of temporarily augmenting the marrow

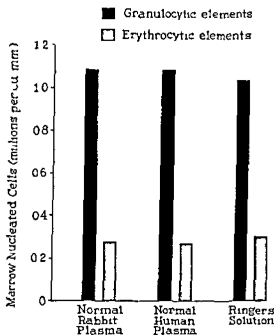


FIGURE 20 Uniformity in the marrow nucleated cell counts at the end of a three week injection period in normal rats given either boiled extracts of normal rabbit or human plasma or Ringer s solution Average counts of eight animals in each group

persist it seems certain that the development of more sensitive methods of detecting the erythropoietic factors will firmly establish their presence in normal blood Therefore it may be assumed that the plasma erythropoietic factors are responsible for the maintenance of normal erythrocytic equilibrium in addition to determining the erythropoietic responses to stimuli arising from physiologic and pathologic changes

Utilizing existent experimental data it is possible to formulate a hypothesis concerning the primary regulatory control of erythropoiesis It has been suggested that erythropoiesis consists of three basic physiologic processes of which only two appear to be under the influence of humoral regulatory mechanisms (see page 81) As depicted schematically in Figure 21 hemoglobin synthesis and maturation of myeloid erythrocytic precursors apparently occur simultaneously and most likely reflect the inherent potential of the

Decreased arterial oxygen saturation, whether secondary to lowered oxygen tension of inspired air or to circulatory or pulmonary defects, will produce tissue hypoxic hypoxia. The latter then induces augmented plasma erythropoietic activity, which increases the peripheral erythroid values and oxygen-carrying capacity of the blood by stimulating myeloid erythrocytogenesis. Depending on the severity of the hypoxemia, erythropoiesis will be established at a higher level compatible with the maintenance of a normal tissue oxygen supply, or the process will continue unabated in an abortive attempt to restore equilibrium. The unphysiologic polycythemia in patients with certain congenital cardiac defects is an example of the latter situation. If the cause of the decreased arterial oxygen saturation can be eliminated, the erythropoietic stimulatory capacity of the plasma will fall and the erythroid values will return to normal levels.

A reduction in the oxygen-carrying capacity of the blood secondary to blood loss would, through the resultant tissue anemic hypoxia, enhance plasma erythropoietic activity in a similar manner. In the presence of normal myeloid elements, increased red cell production continues until the blood is again capable of fulfilling the oxygen requirements for normal cellular metabolic activity.

The humoral erythropoietic regulatory mechanism may also be implicated in situations involving changes in the overall rate of metabolic activity and oxygen requirements rather than alterations in oxygen supply. Increased need would produce relative tissue hypoxia even in the face of stable erythroid values and normal oxygenation of the blood. In this respect, Jacobson and his co-workers[5, 118] have shown that the administration of the naturally occurring metabolic stimulant triiodothyronine does stimulate erythropoiesis in recipient animals. It may then be inferred that reduced metabolic requirements would produce a situation analogous to transfusion polycythemia or hyperoxia which undoubtedly depress plasma factor activity. Further study should elucidate the likely possibility that such an effect on the plasma erythropoietic factors may be of major importance in the pathogenesis of the lowered erythroid values which accompany various

output it would seem to be of relatively minor importance in the final restoration of erythrocytic equilibrium Acceleration in the rate of release of red cells from the marrow is therefore probably a stop gap measure brought into play in emergency situations until sufficient time has elapsed for the activation of more effective means of enhancing erythropoiesis The latter is undoubtedly accomplished as the direct result of the humoral erythropoietic factors As previously proposed (see Chapter V) the latter through their effects on hemocytoblastic or reticulum cell erythrocytic differentiation and homeoplastic cellular division appear to control respectively the quantity of hemoglobin produced and the number of erythrocytes in which it is distributed In addition it is suggested that the combined activities of both plasma factors govern not only the amount of hemoglobin and the number of red cells formed but also determine the size and hemoglobin content of the individual erythrocytes It may be concluded with experimental support that in the presence of normal myeloid elements the plasma factors constitute the chief and in all probability the only means of significantly altering both quantitatively and qualitatively erythropoietic activity

We concur with the concept of erythropoietic control developed by Jacobson and his associates [318] Tissue oxygen tension or more specifically the relationship between oxygen supply and cellular metabolic requirements can be assumed to control either the actual formation of the factors their release into the circulation changes in activator inhibitor complexes or possibly their activation from inactive precursors A well balanced equilibrium appears to exist for a given level of metabolic activity which ensures under normal conditions not only a steady erythroid state and an oxygen supply commensurate with the tissues needs but also the production of red cells which are of an optimal size and hemoglobin content for the most efficient completion of their physiologic functions This humoral regulatory mechanism apparently responds to alterations in the dynamic relationship between oxygen supply and tissue requirements Erythrocytic equilibrium is thereby re established at the same or a different level as the situation warrants

Chapter VIII

SITE(S) OF PRODUCTION OF THE PLASMA FACTORS

The majority of the observations on sites of production of erythropoietic factors have had negative value. They have tended to eliminate such likely organs as bone marrow, spleen, and liver as places of origin of these materials. Plasma and serum constitute the most potent source of the erythropoietic factors; however, erythrocytogenic agents have been found in urine[18, 60, 61, 16, 18, 9, 1] milk[3] and yellow bone marrow.[133] Scattered reports of activity in other tissues have appeared, but in most of these instances the responses have been equivocal and lack confirmation. At the present time there exists direct and conclusive evidence for erythropoietic stimulatory properties only in the biologic materials listed above.

In view of the findings which indicate that there are two plasma erythropoietic factors with different physical, chemical, and physiologic characteristics, experimental observations may reflect these properties. *Consequently, the type of materials studied and the* methods used to demonstrate erythropoietic activity become important considerations in the interpretation of such data. In addition, the possibility of different sites of production cannot be excluded.

Carnot and Deflandre were the first investigators to study the erythropoietic stimulatory activity of different organs.[107] Aqueous extracts of femoral marrow, brain, and liver of bled rabbits were reported to induce a response in recipient animals similar to "anemic" serum. Extracts of spleen, kidney, intestine (lymphocytic tissue), suprarenal capsule, and muscle were found to be inactive. These workers speculated that the large amounts of blood in organs such as the brain and liver may have accounted for the activity in these tissue extracts and concluded that "hemopoietine" probably existed only in the blood and bone marrow, with perhaps a greater concentration in the latter. However, they sug

hypometabolic states such as hypopituitarism and myxedema Conversely increased calorigenesis may be responsible for the erythrocytosis associated with Cushing's syndrome and the administration of ACTH or the corticosteroids

In summary the primary regulatory mechanism which governs erythropoiesis is most likely dependent on the relationship between the available supply of oxygen and the metabolic requirements of *some as yet undetermined tissue or tissues It is mediated* to myeloid elements by the plasma erythropoietic factors This well balanced mechanism apparently maintains the normal erythroid steady state and is responsive to even minor changes in the existent equilibrium Although this hypothesis is in part speculative it is fortified by many experimental observations Pending further investigation it provides a logical and rational explanation for the physiologic control of the normal erythroid steady state and for the regulatory mechanisms which govern the production of red blood cells in a number of abnormal states

alter an animals ability to produce the humoral factors in response to phenylhydrazine garlic feeding or excessive ultraviolet irradiation However he reported that splenectomy did prevent the formation *of the active factors in animals rendered* anemic by blocking the reticuloendothelial system with India ink or in those exposed to simulated high altitude Donors with impaired *reticuloendothelial systems failed to produce enhanced* activity after being placed in atmospheres of reduced oxygen tension but responded to the production of anemia as manifested by an augmented erythropoietic stimulatory effect of their serum *Tei concluded from these studies that there existed two factors* which were formed at different sites in response to varying stimuli *Recent observations (see Chapter V) are in agreement with the* former postulate but do not support the contention that the method of augmenting a donors plasma activity determines the specificity of the plasma factors so produced The question as to different sites of production has not yet been answered

More recently Mirand and Prentice[35] subjected splenectomized rats to simulated high altitudes and concluded that the spleen was *not responsible for the increased plasma erythropoietic stimula*tory activity induced by hypoxic hypoxia Although seemingly contradictory of Teis observations *it should be pointed out that* the method used by the former investigators to demonstrate erythropoietic activity consisted of the iron 59 uptake in hypophysectomized rats This technique was not available to Tei Jacobson and his associates[119] have shown that splenectomy does not eliminate or alter the capacity of experimental animals to augment their plasma erythropoietic activity following bleeding or the administration of cobalt Neither does removal of the thymus stomach intestines pancreas or 90 per cent of the liver

Bonsdorff and associates[117, 13, 519] *have reported that blood sub*jected to reduced oxygen tension *in vitro* developed increased erythropoietic activity and that normal subjects exhibited accelerated erythropoiesis following venous occlusion of an extremity These observations suggested the possibility that erythropoietin was formed in the blood itself or by myeloid elements Other workers have failed to confirm these somewhat inconclusive findings[79, 80] and recent studies have shown that normal plasma re

gested that the activity of their bone marrow extracts may have been due to the fact that this was the site of the factor s action rather than its formation

More recent experiments have been conducted by Gordon and his co workers[183, 184] in an attempt to detect the site of production of the circulating erythropoietic factor(s) Although their original investigations suggested activity in extracts of liver and bone marrow [183] subsequent studies[184] did not corroborate these findings Boiled extracts of plasma liver spleen thymus lung brain skeletal muscle bone marrow and packed red cells from anemic donors were administered to other animals Only the plasma extracts were found to possess erythropoietic stimulatory properties Erslev [04] has evaluated saline extracts of bone marrow spleen thymus lung and kidneys from anemic animals and failed to detect activity in any of these materials

Most of the other reported observations on the possible site or sites of production of the erythropoietic factors have dealt with an animal s ability to elaborate these substances following damage to or removal of one or more organs Considerable import must be attached to evidence obtained in this manner because the active agents may not be stored but released into the circulation immediately upon formation Even if the factors are present in high concentrations at their place of origin or some storage site not concerned with their elaboration it is equally possible that they may exist there in an inactive form and be activated in some manner by hypoxia concomitantly with their release into the plasma The above circumstances would preclude the demonstration of erythropoietic activity in various tissues even if the organ studied was actively involved in the production of the humoral factors Therefore the failure to detect activity in certain tissue extracts cannot be considered conclusive evidence that the erythropoietic factors are not produced therein or subjected to some measure of regulatory control by that particular tissue

Tei[118] studied the relationship of the spleen and reticuloendothelial system to the humoral erythropoietic factors and reported that splenectomized recipients exhibited greater erythropoietic stimulation following the administration of active sera than did normal animals He also noted that removal of the spleen did not

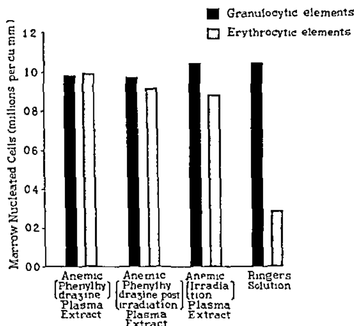


FIGURE 22 Myeloid erythrocytic hyperplasia in normal rats injected daily for three weeks with boiled extracts of plasma from rabbits rendered anemic by phenylhydrazine following exposure to a near 100 per cent lethal dose of total body x irradiation and from other rabbits with anemia secondary to ionizing irradiation alone The nucleated red cell counts in these animals were comparable to those in recipients of phenylhydrazine induced anemic plasma extracts from nonirradiated donors Average cell counts of ten rats given Ringer s solution and four animals in each of the other groups receiving the above described materials (From Linman J W and Bethell F H The effect of irradiation on the plasma erythropoietic stimulating factor *Blood* 12 123 129 1957 Reprinted by permission)

manifested peripherally by erythrocytosis due to increased numbers of microcytes and reticulocytosis The hemoglobins and hematocrits did not show significant change in any group throughout the period of observation

From these findings it would appear that an actively functioning or regenerative bone marrow is not essential for the production of the thermostable plasma erythropoietic factor and that

mains inactive even after equilibration against pure nitrogen for 24 hours [98] There are additional data that tend to negate the possible role of the blood or blood forming organs in the production of the plasma erythropoietic factors

Erslev and Lavietes [11] have carried out experiments regarding the effects of nitrogen mustard on the elaboration of the humoral agent(s) They noted no impairment after treatment with this cytotoxic agent in the ability of rabbits to develop increased plasma erythropoietic activity in response to hemorrhagic anemia We have conducted similar experiments with x irradiation [189] These studies were designed to see if a near 100 per cent lethal dose of total body x ray which immediately arrests erythroblastic cellular division and differentiation would prevent enhanced production of the stimulating factors It was also desired to determine if the anemia produced as a delayed effect of the irradiation itself was a stimulus to plasma factor elaboration even though the donor animals had hypoplastic marrows

Adult New Zealand rabbits weighing 2 5 to 3 0 kilograms were given total body x irradiation (total dose – 750 r filtration – 0 5 mm copper and 1 mm aluminum 245 KV dose rate – 17 6 r per minute) These animals were then divided into two groups One group was started on daily subcutaneous injections of 1 ml of a 2 5 per cent solution of phenylhydrazine twenty four hours later After three to four injections these animals were *anemic with hemoglobins ranging from 2 2 to 6 6 and averaging 3 7 Gm* per cent They were then exsanguinated Boiled and perchloric acid precipitated plasma extracts were prepared according to the method previously described (see page 44) The other animals were followed for two to three weeks after irradiation until their hemoglobins averaged approximately 6 Gm per cent They were then exsanguinated and plasma extracts prepared These materials were given to groups of normal rats in individual doses equivalent to 2 per cent of the recipient s body weight (18 daily injections over a three week period) Other rats were injected with boiled *plasma extracts from nonirradiated rabbits rendered anemic by phenylhy*drazine A control group received comparable amounts of Ringer s solution

The results of this endeavor are in part summarized in Figure 22 which *depicts the average marrow nucleated red cell counts* in each group of rats at the end of the injection period The extracts of plasmas from both irradiated and nonirradiated rabbits with phenylhydrazine induced anemia and from those with anemia secondary to irradiation alone were all capable of stimulating erythropoiesis in normal rats The myeloid erythrocytic hyperplasia was comparable in degree in all of these groups and was

Since tissue hypoxia apparently stimulates the formation or activation of the humoral factors the presence of increased erythropoietic activity in anemic and hypoxemic states where cardiac and pulmonary adjustments maintain the oxygen supply to such organs as the brain suggests that they may be elaborated by some relatively nonvital cellular system or organ Even though experimental observations appear to eliminate a number of organs as loci of erythropoietic factor production it would seem imprudent to exclude them without reservation The number of unknown variables involved in such studies demand that caution be employed in reaching definitive conclusions These include to mention but a few the possibilities that multiple organs may be involved and that the factors may be stored or activated in tissues other than those in which they are formed The unresolved question as to the existence of inhibitor substances further adds to the complexity of this problem

The most persuasive data linking erythropoietic factor production to a specific organ have emanated from the laboratory of Jacobson and his associates [146, 147, 318, 333, 334] These investigators have reported that nephrectomized rats rabbits and mice fail to develop enhanced plasma stimulatory activity in response to hemorrhage the administration of cobaltous chloride or exposure to lowered barometric pressure Their methods of demonstrating erythropoietic activity in recipient animals have included the incorporation of iron 59 in hemoglobin of starved and hypophysectomized rats and the reticulocyte response in transfusion induced polycythemic mice Following bilateral ureteral ligations the augmentation in plasma activity was only slightly less than that observed in the intact controls given cobalt or bled

These workers have concluded from their observations that erythropoietin is most likely produced by the kidneys but suggest that the toxic reaction to nephrectomy may prevent its formation in some other site or it may be formed in the kidney as an inactive precursor and activated elsewhere Animals with intact kidneys were used as recipients so it seems unlikely that the kidneys activate an inactive precursor formed in some other organ The possibility that such a precursor can be activated only by a hypoxic kidney has not been eliminated Since ureteral ligation

it is not formed by hemopoietic elements but by a relatively radiation resistant tissue The observations of Stohlman and Brecher permit a similar conclusion in respect to the humoral factor which stimulates hemoglobin synthesis They noted that exposure of rats to x irradiation prior to bleeding or placing them in atmospheres of reduced oxygen tension not only failed to diminish the enhancing effect of their plasma on the incorporation of iron 59 in hemoglobin of recipient animals but actually potentiated the activity Therefore neither plasma factor seems to be formed by hemopoietic or other tissues possessing comparable radiosensitivity Mathe and Bernard[3 1] have also concluded that irradiation does not interfere with the development of enhanced plasma erythropoietic activity after hemorrhage

An alternative explanation for the failure of nitrogen mustard or x irradiation to prevent the appearance of increased amounts of the erythropoietic factors in response to hypoxia must be excluded It is possible that injury to the myeloid elements may enhance the accumulation of the active agents in the plasma because of an inability of the target tissue to utilize them in blood formation There is little doubt that the level of plasma activity is determined at least in part by the functional integrity of the marrow On the other hand if the factor or factors were produced by the blood forming organs it would seem unlikely that injury severe enough to prevent their utilization could occur without seriously interfering with their formation

The evidence which indicates that the endocrine glands exert a modifying influence on erythropoiesis through their effects on metabolic activity rather than participating in the primary control of red cell production has been discussed (see Chapter II) Extirpation of the orthodox endocrine glands singly or together does not prevent the erythropoietic response to hypoxic stimuli Furthermore enhanced erythropoietic activity has been demonstrated in the plasma of hypophysectomized adrenalectomized thyroidectomized and castrated animals after bleeding[140 141 3] Consequently current data render as untenable the thesis that the endocrine glands may be sites of production of the humoral erythropoietic factors which have been studied

in vivo response to acute hemorrhage as measured by plasma iron turnover Ersley has concluded from these studies that the anemia of uremic rabbits is not related to the presence or absence of normal kidney tissue and is associated with both decreased production and impaired utilization of the plasma factor He does not believe that his observations support the theory that the kidney is the site of formation of the humoral erythropoietic factor(s)

The problem is further complicated by the failure of others to reproduce the findings of Jacobson and his co workers Mirand and Prentice[197 9] using the uptake of iron 59 in hypophysectomized rats as a measure of response have reported that bilateral nephrectomy does not prevent the development of enhanced plasma erythropoietic activity in rats subjected to lowered barometric pressure but does abolish the response to hemorrhage or cobalt They have concluded that erythropoietin" is not produced in the kidneys in response to hypoxic hypoxia However these investigators concur with the thesis that the kidneys are required for the increase in humoral erythropoietic factor activity evoked by hemorrhage or the cobaltous ion

The question as to whether different tissues might respond to different types of hypoxic stimuli cannot be answered However it would seem highly improbable that the effect of hypoxic hypoxia would be qualitatively different from that of hemorrhage Jacobson and his associates[315] have shown that in their hands nephrectomy completely abolishes the response to a low oxygen atmosphere The explanation for these contradictory findings is not yet apparent

There are additional observations on the hemopoietic effects of bilateral nephrectomy in rabbits[330] which indicate that the kidneys are at least not the sole place of origin of the plasma erythropoietic factors Nephrectomized animals do not develop reticulocytosis but neither do their reticulocyte counts fall precipitously The speed with which the plasma regulatory mechanism is capable of altering erythropoiesis providing no myeloid abnormality exists is well documented in many experimental situations involving both increased and decreased humoral stimulatory activity This effect has been demonstrated after time limited exposure to anemic and hypoxic hypoxia[98] and decreased erythropoi

produced a comparable degree of uremia without significantly decreasing the plasma activity evoked by hypoxic stimuli accumulation of waste products would not seem to explain the findings However a negative response by the assay methods used does not necessarily indicate an absence of the erythropoietic factors Nephrectomized animals still may be able to produce a normal or a decreased amount of an active agent which is undetectable by the relatively insensitive methods available to demonstrate erythropoietic activity

Naets[1 37] *has described similar observations in nephrectomized dogs* Following removal of both kidneys the animals were kept alive by peritoneal dialysis He observed a marked decrease in iron 59 incorporation and plasma turnover in these animals whereas similar measurements in dogs subjected to bilateral ureteral ligation were virtually normal In addition Naets has described a disappearance of the erythroblasts from the marrows of nephrectomized dogs which was evident in three to six days after extirpation of the kidneys Erythroblasts were stated to persist in the marrows of dogs after ureteral ligations Erythroblastopenia has *also* been reported in persons *with* acute anuria but not *in* patients with chronic uremia even though the level of the NPN was comparable[38] Naets has concluded from these findings that bilateral nephrectomy abolishes erythropoiesis in the dog

Erslev[09 39] has also carried out studies designed to evaluate the role of the kidneys in the formation of the plasma erythropoietic factor(s) but with different results He reported that rabbits subjected to bilateral nephrectomy or ureteral ligation or section and bled in the preuremic phase showed a low level of erythropoietic factor activity as determined by the reticulocyte response *in recipients of their serum Although he did not observe this find*ing or an increase in reticulocytes or bone marrow erythrocytic elements in severely uremic rabbits following hemorrhage myeloid erythropoiesis remained active despite the complete absence of renal tissue The serum of uremic rabbits did not impair the erythropoietic activity of serum from normal bled animals and the uremic rabbits did not respond to an infusion of serum from anemic but otherwise intact animals *He has also reported that* both bilateral nephrectomy and ureteral ligations abolish the

At the present time the possible place of origin of the thermostable ether soluble plasma erythropoietic factor is entirely conjectural In view of the similarities between the chemical characteristics and erythropoietic effects of this substance and batyl alcohol (see Chapter VI) yellow bone marrow deserves further study

In summary experimental observations permit a number of tentative conclusions regarding the site or sites of production of the plasma erythropoietic factors There exists evidence that tends to exclude the following organs or tissues Myeloid hemopoietic elements liver spleen thymus lung brain skeletal muscle mature erythrocytes pituitary thyroid adrenals gonads lymphoid tissue stomach intestines and pancreas The kidneys are most likely involved in some manner in the humoral erythropoietic regulatory mechanism It is suggested that the plasma erythropoietic factors may have different sites of production The place of origin of the thermostable ether soluble factor which stimulates erythroblastic cellular division is unknown The relatively thermolabile ether insoluble factor which appears to be a mucoprotein and augments hemoglobin synthesis may be elaborated by the kidneys The possibilities that the erythropoietic factors may be stored in sites other than their formative tissue(s) produced as inactive precursors in one organ and activated elsewhere or exist in the form of activator inhibitor complexes cannot be excluded

etic activity occurs promptly in transfusion induced polycythemic animals[1 9] It has also been shown that exposure of rabbits to an atmosphere of 68 per cent oxygen is followed by a much more rapid decline in the reticulocyte count than is the removal of both kidneys[5] If the integrity of the humoral erythropoietic regulatory mechanism was entirely dependent on the kidneys it would seem that bilateral nephrectomy should be followed by a prompt and essentially complete cessation of reticulocyte production In addition normal or even increased numbers of erythrocytic precursors have been observed in the marrows of rabbits three days after bilateral nephrectomy[330] This finding is in distinct contrast to the absence of marrow erythroblastic elements in nephrectomized dogs described by Naets[3 -3 7]

Further studies are needed before the exact role of the kidneys in the determination of plasma erythropoietic factor activity can be accurately defined However available data make it difficult to envision the kidneys as the only site of formation or activation of the humoral agents On the other hand the elaboration of one plasma erythropoietic factor by the kidneys cannot be excluded and could explain some of the variations in reported experimental observations The studies of Jacobson and his associates[146 147 318 3 3 3 4] strongly suggest that this is true On the basis of their test materials and methods of demonstrating activity (iron 59 incorporation in hemoglobin) it would appear to be the relatively thermolabile factor which stimulates hemoglobin production Continued elaboration of the thermostable ether soluble factor might then account for the persistence of peripheral reticulocytes and myeloid erythrocytic precursors in totally nephrectomized animals

The frequent occurrence of anemia in patients with chronic renal disease[331 333] provides further support for active participation by the kidneys in the regulatory control of erythropoiesis Conversely the occasional association of polycythemia with various renal lesions (tumors polycystic disease and hydronephrosis)[334 336] is also compatible with such a thesis In addition erythrocytosis has been reported to follow the partial occlusion of a renal vein in dogs[337] Future investigation should clarify this apparent relationship between the kidneys and erythropoiesis

Normal human plasma extracts do not induce clear cut erythropoietic stimulation in recipient animals when administered in the usual doses However evidence has been obtained[149, 190] which indicates that erythropoietic factors do exist in normal human plasma Activity is apparently present under normal conditions but escapes detection unless the plasma extracts are concentrated approximately tenfold This finding together with others such as the depressant effect of hyperoxia on erythrocytogenesis[84, 8] supports the concept that the plasma factors exert important physiologic control over erythropoiesis and probably constitute the primary mechanism responsible for the maintenance of the normal erythroid steady state in man It also may be concluded from available data that the relationship between oxygen supply and tissue requirements controls the formation or activation of the plasma erythropoietic factors in the human species The humoral agents then mediate the specific requirements for erythrocyte production to the bone marrow and thereby automatically establish a level of erythropoiesis commensurate with the oxygen needs for normal cellular metabolic activity

Observations have been carried out on the erythropoietic stimulatory capacity of plasmas from patients with a number of primary and secondary anemias However failure to observe erythropoietic activity in certain of these plasmas or sera merely indicates an erythropoietic effect equal to or possibly less than that present normally Therefore it is not yet possible to classify anemias on the basis of decreased plasma erythropoietic factor activity On the other hand enhanced activity has been demonstrated in a variety of disorders including pernicious anemia acute and chronic hemorrhagic anemia acquired and hereditary hemolytic anemias hereditary leptocytosis sicklemia acute and chronic leukemia erythroblastosis fetalis Hodgkin s disease metastatic carcinoma and hypoplastic anemia[1, 3, 1, 5, 134, 149, 15, 18, 190, 191, 00, 19, 1, 8]

The plasma erythropoietic factors undoubtedly play an important role in all anemias even though certain deficiency states or abnormalities of myeloid elements are capable of altering red cell formation For the purpose of this discussion it would seem desirable to divide the anemias into the following groups

Chapter IX

ROLE OF THE ERYTHROPOIETIC FACTORS IN MAN

Prior to the demonstration of erythropoietic activity in heat denatured plasmas and the development of short term assay methods observations on the humoral factors in man were necessarily restricted and in most instances indirect The earliest mention of their possible role in man is found in the reports of Carnot and Deflandre[106, 107] These investigators described increases in the red cell counts of anemic patients given serum obtained twenty hours after bleeding but did not specify the type of anemia treated or the source of the serum

In 1939 Loeschcke and Schwartzer[309] reported that both the serum of a man residing at high altitude and human umbilical cord blood evoked erythrocytosis in normal rabbits Several years later Bonsdorff and Jalavisto[117] immunized rabbits against human plasma and noted evidence of erythropoietic stimulation in such animals given plasma from patients with congestive heart failure and stagnant hypoxia Oliva and associates[134] utilized normal subjects as recipients in their studies in 1949 on the erythropoietic activity of human anemic plasmas In addition observations on patients with regional hypoxia[96, 97] and generalized myeloid erythrocytic hyperplasia suggested that a humoral factor was active in the human species

The existence of a humoral erythropoietic regulatory mechanism in man has now been confirmed There is excellent support for the conclusion that the chemical and physiologic characteristics of the human erythropoietic factors are identical with those in other mammals The assumption that the information obtained through animal experimentation is directly applicable to man is a valid one Therefore these basic principles will be utilized to supplement the existent observations on the role of humoral factors in the control of erythropoiesis in man

in clarifying the role of the plasma erythropoietic factors in the various anemic states

Following acute hemorrhage in an otherwise normal individual with adequate iron stores the restoration of erythrocytic equilibrium is undoubtedly the direct result of the accelerated erythropoiesis occasioned by the enhanced plasma erythropoietic factor activity Hemolysis also induces augmentation in plasma activity but depending on the severity and persistence of the hemolytic process the increased production of erythrocytes may not be adequate The depressed erythroid values which usually accompany hemolysis regardless of the underlying cause probably reflect the ceiling imposed by the functional reserve or capacity of the marrow rather than any abnormality in the humoral erythropoietic regulatory mechanism Therefore it may be logically inferred that the plasma factors by evoking accelerated erythrocytogenesis temper the severity of a hemolytic disorder as reflected by the degree of anemia present It is also possible that they may in some instances produce "compensated disease without anemia The latter occurrence has been mentioned as being incompatible with the theory that hypoxia is the primary erythropoietic stimulus (see page 121) However in view of the delicately balanced equilibrium which apparently exists it would not seem unreasonable to surmise that minor and perhaps undetectable changes in circulating hemoglobin levels may sufficiently enhance plasma factor activity so that red cell production exceeds peripheral destruction More sensitive methods capable of accurately quantitating changes in plasma erythropoietic activity will be needed to clarify this problem

The plasma erythropoietic factors cannot be implicated in the production of the anemias due to blood loss However it may be considered axiomatic that their increased elaboration is vital for the restoration of normal values In the face of continued hemolysis or bleeding they serve to at least partially counteract the peripheral loss until it can be eliminated or brought under control "Aplastic crises have been described in many types of hemolytic disease and have usually been explained on the basis of "marrow exhaustion It is conceivable that some failure or defect in the humoral regulatory mechanism may be responsible at least in

- I Increased removal of red cells
  - A Hemorrhage
  - B Hemolysis
- II Impaired blood formation
  - A Deficiencies—iron vitamin B_{12} folic acid etc
  - B Destruction of myeloid elements—hypoplastic and aplastic anemia
  - C Diversion of growth potential—granulocytic leukemia
  - D *Displacement* or *crowding out of marrow elements*—myelophthisic anemia
  - E Defective erythropoiesis—hereditary hemoglobinopathies
  - F *Depression (etiology unknown)*—*chronic infection* renal insufficiency etc

All anemias are basically the result of inadequate blood formation but the above two categories differ markedly in respect to the pathophysiologic processes responsible for the lowered erythroid values Since all of the metabolic building blocks required for normal red cell formation are present in otherwise uncomplicated anemic states due to increased removal of erythrocytes from the circulation the marrow is capable of responding However the myeloid erythrocytic hyperactivity may not be adequate and anemia occurs when erythrocyte production fails to keep pace with the peripheral loss In the case of the anemias due to impaired blood formation the primary cause is defective erythrocytogenesis The number of red cells formed by the marrow is insufficient to replace those lost by normal aging For this reason the role of the plasma erythropoietic factors in these different anemic *states will depend on the functional integrity of the myeloid reticulum*

Unfortunately no etiologic classification of anemias is absolute *It is impossible to* delineate all *anemic* states on the basis of a single pathogenetic mechanism The end result often reflects both increased removal and impaired formation of the erythrocytes Chronic blood loss with its resultant iron deficiency and subsequent impairment in hemoglobin synthesis is one of many examples of multiple causes for a measurable lowering of the erythroid values However differentiation as to basic cause is a definite aid

uremia is associated with both decreased formation and impaired utilization of the plasma erythropoietic factor(s).[3,9,339] Gurney and his co-workers[90] have reported their failure to demonstrate enhanced erythropoietic activity in the plasmas of seven patients with refractory anemia secondary to chronic renal disease. Gallagher and his associates[339] described similar findings in fifteen out of the sixteen uremic patients with anemia that they studied. It has also been suggested that congenital hypoplastic anemia may be due to a deficiency of an essential plasma factor.[135] Many investigators are now working on this aspect of the humoral control of erythropoiesis. Additional information concerning the plasma erythropoietic factor activity in patients with a variety of refractory anemias should soon be available.

Any discussion of the possible therapeutic potentialities of the plasma erythropoietic factors is perhaps premature. However, since one of the ultimate goals of research on the humoral control of erythropoiesis is to isolate and synthesize an erythropoietic substance or substances which may be effective in the treatment of certain refractory anemias, this subject is of great interest. On theoretical grounds it would seem very unlikely that the plasma erythropoietic factors would be of value in the treatment of otherwise uncomplicated anemias due to blood loss, hemolysis, or impaired erythrocytogenesis secondary to well-established myeloid deficiencies or defects. These conditions are not the result of abnormalities in the humoral erythropoietic regulatory mechanism. Such patients already have increased plasma stimulatory activity due to endogenous elaboration of the factors. Under these circumstances it is highly improbable that the administration of additional amounts of the erythropoietic factors from exogenous sources would be of any therapeutic benefit.

On the other hand, if some anemic states are found to be associated with decreased plasma activity, the humoral factors may constitute specific therapy. It is of interest that the disorders most likely to be associated with depressed plasma activity, e.g., uremia, rheumatoid arthritis, and chronic infection, are the ones in which the anemia has on occasion responded to therapeutic doses of cobalt. This substance has been shown to enhance endogenous plasma erythropoietic activity. However, the erythropoietic re-

part for these occurrences Further studies in this regard are needed

The pathophysiologic activity of the plasma erythropoietic factors in the anemias due to impaired blood formation is more speculative If the prerequisites for normal erythropoiesis do not exist it is obvious that the marrow cannot respond in a normal manner to the stimulus imparted by the humoral erythropoietic factors Since hemopoietic elements are apparently not involved in the elaboration of the plasma factors[189, 11] it then follows that enhanced plasma activity secondary to tissue anemic hypoxia should be *demonstrable in the anemias resulting from either quantitative* or qualitative marrow abnormalities The latter would include deficiencies of vitamin B_1 folic acid or iron a decrease in normal marrow elements secondary to their destruction by physical or chemical means crowding out by foreign tissue or diversion of growth potential by leukemic transformation and defective erythropoiesis resulting from hereditary or inborn abnormalities in hemoglobin synthesis Although the numbers of patients studied have been small enhanced plasma activity has been demonstrated in each of the above hematologic disorders Even though the primary marrow abnormality prevents the normal erythropoietic response to the humoral factors it cannot be assumed that these agents are devoid of pathophysiologic significance Their presence is probably essential for the continued attempt on the part of the marrow to produce some erythrocytes under these conditions and for the restoration of normal erythroid values after the specific marrow deficiency or defect is corrected

The plasma erythropoietic factors are not of pathogenetic importance in these well defined anemias However the very real possibility exists that some abnormality of the humoral erythropoietic mechanism may be of etiologic significance in certain other poorly understood anemias Such anemic states might include among others those which accompany renal insufficiency chronic infection rheumatoid arthritis chronic liver disease and malignancy

The evidence which implicates the kidneys in the humoral control of erythropoiesis has been described (see page 139) It has been postulated, on the basis of animal experimentation that

uremia is associated with both decreased formation and impaired utilization of the plasma erythropoietic factor(s)[310, 339] Gurney and his co-workers[90] have reported their failure to demonstrate enhanced erythropoietic activity in the plasmas of seven patients with refractory anemia secondary to chronic renal disease Gallagher and his associates[339] described similar findings in fifteen out of the sixteen uremic patients with anemia that they studied It has also been suggested that congenital hypoplastic anemia may be due to a deficiency of an essential plasma factor[13] Many investigators are now working on this aspect of the humoral control of erythropoiesis Additional information concerning the plasma erythropoietic factor activity in patients with a variety of refractory anemias should soon be available

Any discussion of the possible therapeutic potentialities of the plasma erythropoietic factors is perhaps premature However since one of the ultimate goals of research on the humoral control of erythropoiesis is to isolate and synthesize an erythropoietic substance or substances which may be effective in the treatment of certain refractory anemias this subject is of great interest On theoretical grounds it would seem very unlikely that the plasma erythropoietic factors would be of value in the treatment of otherwise uncomplicated anemias due to blood loss hemolysis or impaired erythrocytogenesis secondary to well established myeloid deficiencies or defects These conditions are not the result of abnormalities in the humoral erythropoietic regulatory mechanism Such patients already have increased plasma stimulatory activity due to endogenous elaboration of the factors Under these circumstances it is highly improbable that the administration of additional amounts of the erythropoietic factors from exogenous sources would be of any therapeutic benefit

On the other hand if some anemic states are found to be associated with decreased plasma activity the humoral factors may constitute specific therapy It is of interest that the disorders most likely to be associated with depressed plasma activity e g uremia rheumatoid arthritis and chronic infection are the ones in which the anemia has on occasion responded to therapeutic doses of cobalt This substance has been shown to enhance endogenous plasma erythropoietic activity However the erythropoietic re

sponses attributable to cobalt are relatively infrequent and leave much to be desired This fact is not incompatible with the concept that decreased plasma erythropoietic activity may be of pathogenetic significance in some of these anemic states Cobalt probably exerts its erythropoietic effect by producing tissue hypoxia (see page 119) and patients with these refractory anemias are already hypoxic because of the reduced oxygen carrying capacity of the blood If a plasma factor deficiency does exist it would therefore reflect a defect in the patient s ability to respond in a normal fashion by the development of enhanced humoral erythropoietic activity It is not necessary to assume that increased tissue hypoxia i e histiotoxic hypoxia superimposed on the preexistent anemic hypoxia would overcome this hypothetical block in the elaboration or activation of the erythropoietic factors

Since a concentrated and potent source of the erythropoietic factors is not available few attempts have been made to assess their therapeutic effectiveness Beneficial responses although slight have been described in two patients with congenital hypoplastic anemia given five to eight daily 300 ml infusions of plasma from bled human donors [13] However Lubby and his associates[130] were unable to detect an erythropoietic response in a patient with hypoplastic anemia given over a period of nine days a total of 735 ml of erythropoietically active plasma from a patient with hereditary leptocytosis These workers also described negative results in an infant with the anemia of prematurity given anemic dog plasma orally and in another infant with mild erythroblastosis fetalis fed urine from an anemic donor with leukemia Reticulocytosis and a sharp increase in iron 59 uptake have been reported in an uremic woman with anemia who was given 3000 ml of plasma from patients with hemorrhagic anemia and hereditary spherocytosis over a period of *five days* [1 3]

Polycythemia vera and erythrocytosis due to arterial hypoxemia are the hematologic disorders in man which with the exception of temporal factors or the duration of the erythropoietic stimulation most closely simulate the experimentally induced accelerated erythropoiesis observed in normal rats injected with the plasma erythropoietic factors These conditions are associated with an imbalance in the normal erythrocytic equilibrium and in

creased erythropoietic activity maintains the erythroid values at greater than normal levels Enhanced erythropoietic activity is demonstrable in the plasma of patients with both polycythemia vera and secondary polycythemia [189, 19 ... 7, 30 ... 9]

Examples of the myeloid erythrocytic hyperplasia demonstrable in normal rats injected with boiled extracts of plasmas from patients with polycythemia vera and secondary polycythemia are

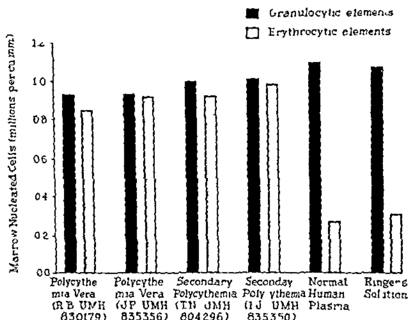


FIGURE 23 Myeloid erythrocytic hyperplasia in normal rats injected daily for three weeks with the boiled extracts of human polycythemic plasmas Patients R B and J P had typical untreated polycythemia vera with hematocrits of 63 and 72 volumes per cent respectively T N had congenital heart disease with a hematocrit of 85 and I J had a pulmonary arteriovenous fistula with a hematocrit of 70 5 Average marrow cell counts of four rats injected with each of the polycythemic plasma extracts eight given a normal human plasma extract and sixteen receiving Ringers solution (From Linman J W and Bethell, F H The plasma erythropoietic stimulating factor in man Observations on patients with polycythemia vera and secondary polycythemia *J Lab and Clin Med* 49 113 127 1957 Reprinted by permission)

shown in Figure 23 The erythropoietic responses in these recipients were similar to those observed following the administration of anemic rabbit plasma extracts and consisted of erythrocytosis due to the presence of numerous microcytes and reticulocytosis without change in the hemoglobin or hematocrit levels These extracts which had been boiled for thirty minutes also failed to enhance iron 59 incorporation in hemoglobin of other rats However plasmas processed by boiling for five minutes or less from patients with polycythemia vera and secondary polycythemia do increase the uptake of iron 59 in erythrocytes[149, 150] and the total red cell volume[19] of recipient rats

The overproduction of the humoral factors in patients with secondary polycythemia is not surprising in view of the well established relationship between hypoxia and plasma erythropoietic activity The decreased arterial oxygen saturation and the resultant tissue hypoxic hypoxia is undoubtedly the stimulus to the increased elaboration of the plasma factors in these patients The marrow responds by increasing the output of erythrocytes in an attempt to restore the normal relationship between oxygen supply and tissue requirements Minor reductions in pO_2 may be compensated for in this manner with the restoration of erythrocytic equilibrium at a higher level However the severity of the arterial hypoxemia may be such that this normal physiologic corrective mechanism is incapable of counteracting the tissue hypoxia Under these circumstances myeloid erythrocytic hyperactivity continues and eventually produces an unphysiologic secondary polycythemia It can be concluded that the plasma erythropoietic factors are directly responsible for the secondary erythrocytosis seen in patients with decreased arterial oxygen saturation whether due to circulatory defects or pulmonary disease The increased erythropoiesis in subjects exposed to lowered atmospheric oxygen tension[340] is apparently the result of a similar mechanism

The presence of increased amounts of the erythropoietic stimulating factors in the plasma of patients with polycythemia vera in the absence of anemia or demonstrable hypoxia raises several interesting questions Polycythemia vera was initially described by Vaquez[441] in 1892 and established as a specific disease entity a

few years later by Saundby and Russel[342] and by Osler[343] Although the clinical manifestations and natural course of the disease have been clearly defined[344-345] its etiology remains unknown The more commonly held theories of causation include hypoxia an abnormality of the hypophyseal hypothalamic system a neoplastic process or a derangement in the physiologic regulatory mechanism responsible for the maintenance of normal erythrocytic equilibrium In view of the potent erythropoietic stimulus imparted by hypoxia its possible role in the pathogenesis of polycythemia vera has been especially stressed However there is no proof that a hypoxic state exists in this disorder and arterial[346] and sternal marrow[349, 350] blood oxygen saturations are normal A possible defect in gas transfer in the pulmonary alveoli has been proposed but there is no evidence to support such a mechanism[351] It has also been shown by Lawrence and his associates[352] that the inhalation of 50 per cent oxygen depresses the plasma iron turnover and red cell iron uptake in secondary polycythemia but not in polycythemia vera The possibility that bone marrow vascular lesions may produce localized hypoxia as suggested by Reznikoff and his co workers[353] cannot be entirely excluded However many clinical observations including the splenomegaly thrombocytosis and leukocytosis so commonly associated with polycythemia vera indicate a different pathogenetic mechanism than simple hypoxia

Polycythemia has been observed in association with lesions in the brain and especially in the region of the pituitary and the midbrain[354-359] but abnormalities of the hypophyseal hypothalamic system have not been demonstrated in patients with the characteristic changes of polycythemia vera Although the midbrain most likely exerts some modifying effect on erythropoiesis there is no convincing evidence implicating the central nervous system in the pathogenesis of polycythemia vera

The neoplastic concept is supported by the increased incidence of leukemia and other related myeloproliferative disorders in these patients However the natural history of this disease is against it being a malignant process It is not rapidly progressive or uniformly fatal unless some complication intervenes Furthermore

the pathologic changes are not those of organ infiltration and abnormal proliferation of a single immature cell type

The theory that polycythemia vera may be due to some derangement *in the physiologic* mechanism which maintains the erythroid steady state in the past considered by most to be speculative is supported by the finding of increased erythropoietic activity in the plasma of these patients Whereas the enhanced plasma erythropoietic activity in subjects with secondary polycy

TABLE XIII

ERYTHROCYTOSIS IN RATS INJECTED FOR 2 WEEKS WITH THE BOILED EXTRACTS OF PLASMAS OBTAINED FROM PATIENTS WITH POLYCYTHEMIA VERA BEFORE AND AFTER TREATMENT WITH P[3] MEAN COUNTS (MILLIONS PER CU MM) OF 6 ANIMALS IN EACH GROUP (From Linman J W Bethell F H and Long M J Factors controlling hemopoiesis Experimental observations on their role in polycythemia vera *Ann Int Med 51* 1003 1018 1959 Reprinted by permission)

Source of Plasma Extract	*Baseline*	*1 Week*	*2 Weeks*
T C —VARH 6738 Pre treatment (1)	7 39	8 30	8 98
T C —VARH 6738 *Post treatment (2)*	7 35	9 07	9 87
E S —VARH 8069 Pre treatment (3)	7 19	8 30	8 64
E S —VARH 8069 Post treatment (4)	7 05	8 21	8 80
Normal Human Subject	7 47	7 37	7 37

Donor s Erythroid Values

(1) Hgb 19 5 gms per cent
Hemat 63 5 vols per cent
RBC 7 500 000 per cu mm

(2) Hgb 14 2 gms per cent
Hemat 49 5 vols per cent
RBC 5 060 000 per cu mm

(3) Hgb 18 2 gms per cent
Hemat 63 5 vols per cent
RBC 8 500 000 per cu mm

(4) Hgb 15 2 gms per cent
Hemat 45 0 vols per cent
RBC 5 550 000 per cu mm

themia can be logically explained on the basis of hypoxia increased amounts of these humoral agents are present in patients with polycythemia vera in the absence of demonstrable hypoxia In addition the augmented plasma factor activity persists regardless of the administration of specific therapy designed to depress erythropoiesis

The erythrocytosis and reticulocytosis induced in normal rats by the boiled extracts of plasmas procured from two patients with polycythemia vera before and after treatment with radioactive phosphorus are shown in Table XIII and Figure 24 There were

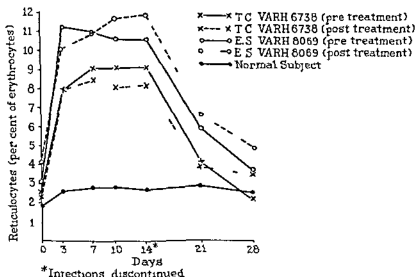


Figure 24 Reticulocytosis in normal rats injected with the polycythemic plasma extracts described in Table XIII Average counts of six animals receiving each of the above described materials (From Linman J W Bethell F H and Long M J Factors controlling hemopoiesis Experimental observations on their role in polycythemia vera *Ann Int Med* 51 1003 1018 1959 Reprinted by permission)

no associated increases in hemoglobins or hematocrits in these animals (Table XIV) and microcytes with decreased osmotic resistance were demonstrable Myeloid erythrocytic hyperplasia was also evident at the end of the two week treatment period

TABLE XIV

Stability of the Hemoglobins (Grams Per 100 Ml) and Hematocrits (Volumes Per Cent) in Recipients of the "Polycythemic Plasma Extracts which Induced the Erythrocytosis Shown in Table XIII
Average Values of 6 Rats in Each Group

Source of Plasma Extract	*Determination*	*Baseline*	*2 Weeks*
T C—VARH 6738 Pre treatment	Hgb Hemat	15.3 42.9	14.8 40.6
T C—VARH 6738 Post treatment	Hgb Hemat	16.4 45.3	15.7 44.6
E S—VARH 8069 Pre treatment	Hgb Hemat	14.2 39.1	14.1 40.1
E S—VARH 8069 Post treatment	Hgb Hemat	13.6 37.8	13.7 37.3
Normal Human Subject	Hgb Hemat	14.5 42.0	14.2 41.2

in the rats injected with the polycythemic plasma extracts (Fig 25) Enhanced plasma erythropoietic activity persisted in both of these patients after therapeutic remission had been achieved and at a time when their erythroid values were entirely normal To date we have studied the plasmas of sixteen patients with active polycythemia vera and eight in therapeutic remission Identical responses have been observed in normal rats given multiple daily injections of the thermostable or other soluble fractions of each of these plasmas Augmented amounts of the relatively thermolabile factor which enhances hemoglobin synthesis were also demonstrable in these plasmas when tested in the unmodified state or after boiling for less than five minutes

Since the plasma erythropoietic factors do not appear to be a by product of hyperactive myeloid elements considerable significance must be attached to the presence in patients with polycythemia vera of enhanced plasma activity which persists irrespective of the institution of myelosuppressive therapy and occurs

tients with polycythemia vera but the humoral agent which controls erythroblastic cellular division apparently predominates Microcytes are evident in their peripheral blood[360, 61] and demonstrable graphically by Price Jones measurements (Fig 26) Fragility determinations utilizing the direct cell enumeration technique reveal decreased resistance of the microcytes to lysis in hypotonic media (Fig 26) The abnormalities are still discernible during therapeutic remissions (Fig 27) These cells are

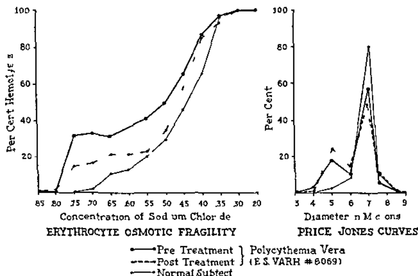


FIGURE 27 Erythrocyte osmotic fragilities measured by a direct cell enumeration technique and red cell diameter distributions of a patient with polycythemia vera The microcytes with decreased resistance to lysis in hypotonic media persisted in this patient as did enhanced plasma erythropoietic activity (Table XIII Figs 24 and 25) after treatment with P[32] Control values are the composite curves of eight normal subjects (From Linman J W Korst D R and Bethell F H Some observations on the stimulation of erythropoiesis by humoral factors *Ann New York Acad Sc* 77 Art 3 638 649 1959 Reprinted by permission)

remarkably similar to those observed in normal rats given the thermostable ether soluble plasma erythropoietic factor (Figs 8 and 10 pages 55 and 57) As noted in the recipients of boiled

This finding is in agreement with those which have shown that irradiation does not interfere with erythropoietic factor elaboration [189, 6]

Although we have observed enhanced plasma erythropoietic activity *in all patients with polycythemia vera studied so far* others have not Contopoulos and his associates[19] reported increased activity in most but not all such plasmas that they studied Gordon has also described negative results [1, 3] More than one form of this disease may exist However varying degrees of increased activity perhaps undetectable by the relatively insensitive assay methods available would seem more likely since the severity of the disease varies clinically

Augmented amounts of both the thermostable and relatively thermolabile plasma erythropoietic factors are present in pa

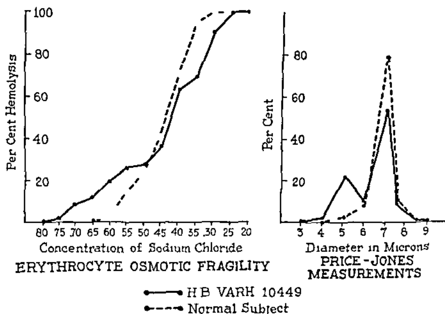


FIGURE 26 Erythrocyte osmotic fragility determined by a direct cell enumeration technique and Price Jones measurements of a patient with active polycythemia vera (Hgb 18 2 gm per cent Hemat 60 vol per cent and RBC—6 460 000 per cu mm) These findings are similar to those observed in normal rats injected with boiled extracts of anemic rabbit plasma (Figs 8 and 10)

cursors was unaffected by the humoral stimulus increased production of normal red cells should result

The small cells with abnormal osmotic behavior detectable in patients with polycythemia vera would certainly appear to reflect a lack of parallelism between the activity of the thermostable ether soluble plasma erythropoietic factor and the humoral agent which enhances hemoglobin synthesis This finding assumes added import in the light of evidence which suggests a possible relationship between the former factor and batyl alcohol The latter is capable of exerting a stimulatory effect on the proliferation of all myeloid elements Consequently experiments were designed to test the hypothesis that the leukocytosis and thrombocytosis so commonly observed in patients with polycythemia vera might also be the result of humoral factor activity These studies indicate that the ether soluble or thermostable fractions of plasmas from such patients do possess thrombopoietic and granulopoietic activity [379]

Plasmas from three patients with active polycythemia vera and three with normal erythroid values subsequent to treatment with radioactive phosphorus were lyophilized and extracted with reagent ether The residue remaining after the ether was removed was reconstituted to the original volume of the plasma with distilled water Each plasma extract was tested in groups of normal rats The recipients of 10 injections of these polycythemic plasma extracts in daily doses equivalent to two per cent of the rats body weights manifested typical erythrocytic responses i e erythromicrocytosis reticulocytosis and myeloid erythrocytic hyperplasia without increases in their hemoglobin or hematocrit determinations In addition these animals developed thrombocytosis which although not marked was a consistent finding in all animals receiving the ether soluble fractions of these polycythemic plasmas (Table XV) The thrombocyte counts returned promptly to normal levels in those animals not killed for marrow studies after the injections were stopped Leukocytosis was not observed in these rats (Table XV) and myeloid granulocytic hyperplasia was not present However their marrow nucleated red cell counts increased and megakaryocytes were very numerous The normal human plasma extract was erythropoietically

or ether extracts of anemic plasmas (see page 56) simultaneous osmotic fragility measurements in these polycythemic patients with a quantitative photocolorimetric technique were not significantly at variance with those of normal control subjects These findings indicate that the erythrocytes in patients with polycythemia vera can be divided into two groups made up respectively, of normal cells and microcytes with impaired viability

Berlin and his co workers[36] have described studies with a C^{14} methyl labeled glycine technique which also indicate that the red cells of patients with polycythemia vera can be divided into two classes One has a normal survival time and the second a life span of only a few days These investigators concluded that the rapid turnover of the short lived erythrocytes is largely responsible for the five fold increase in plasma iron turnover in these patients[363 364] Although most descriptions of the erythrocytes in patients with polycythemia vera fail to mention any significant anisocytosis microcytes have been noted[365] and were emphasized by Price Jones[1] Evidence of increased blood destruction as suggested by bilirubinemia and increased urobilinogen in the urine and feces has also been described[366] and a slight increase in the reticulocytes is fairly common Clear cut abnormalities in osmotic behavior have not been evident by the usual techniques for determining erythrocyte osmotic fragility but a lengthening of the resistance span has been reported with initial hemolysis appearing in more concentrated solutions and complete hemolysis in more dilute media than is normal[35 367 368]

The demonstration of microcytes with decreased osmotic resistance in the blood of patients with polycythemia vera lends further support to the thesis that there are two humoral factors with different modes of action The possibility that the responses observed in normal rats injected with boiled "anemic or "polycythemic plasma extracts might represent an abnormal or unphysiologic effect due to denaturation and subsequent alteration of a single stimulating factor by the processing procedures is untenable in view of the similar type of erythropoietic activity in patients with polycythemia vera If erythroblastic cellular division and hemoglobin synthesis were controlled by a single humoral factor or if the rate of multiplication of myeloid erythrocytic pre

TABLE XV (Continued)

AVERAGE MARROW NUCLEATED CELLS PER CU MM OF 3 RATS IN EACH GROUP AT THE END OF THE INJECTION PERIOD

	Granulocytic	Erythrocytic
1)	962 965	962 455
2)	964,361	1 039 646
3)	916 209	1 078 293
4)	1 008 570	1 096 517
5)	1 053 988	1 447 074
6)	1 036 858	1 037 635
7)	917 609	324 9[illegible]1
8)	915 700	2[illegible]8 155

inactive but did appear to exert a minimal thrombopoietic effect (Table XV)

Since the studies with batyl alcohol indicated a dose response relationship (see Chapter VI) other experiments were carried out to determine if larger amounts of polycythemic plasma extracts might exert a leukocytosis promoting effect in normal rats Plasmas from four additional patients with active polycythemia vera were used All of these patients had increased erythroid values and moderate to marked thrombocytosis Leukocytosis was present in three of these donors The pH of each plasma was adjusted to 5 5 and it was boiled over a direct flame for thirty minutes The filtrates were reconstituted by the addition of distilled water to half of the original volumes of the plasma Normal plasma was processed in a similar manner The plasma extracts were given to groups of normal rats in daily doses calculated on the basis of 2 ml per 100 Gm of body weight but equivalent in terms of the original volume of the plasma to twice those amounts previously used These animals again exhibited typical erythrocytic responses Thrombocytosis was evident and normal plasma once more appeared to exert a slight thrombopoietic effect (Fig 28)

Leukocytosis was also present in these recipients of larger doses of "polycythemic plasma extracts (Fig 28) and was chiefly due to a relative and absolute neutrophilia The constancy of the leukocytosis in all animals and the rapid return of the leukocyte counts to normal after the injections were stopped contribute to

TABLE XV

THROMBOCYTES (x 10^6) AND LEUKOCYTES (x 10^3) PER CU MM IN RATS INJECTED WITH THE ETHER SOLUBLE FRACTIONS OF PLASMAS FROM 6 PATIENTS WITH POLYCYTHEMIA VERA THE THROMBOCYTOSIS WAS ACCOMPANIED BY ERYTHROMICROCYTOSIS RETICULOCYTOSIS AND MYELOID ERYTHROCYTIC HYPERPLASIA MEGAKARYOCYTES WERE ALSO INCREASED MARROW GRANULOCYTIC CELL COUNTS WERE NOT SIGNIFICANTLY ALTERED DAILY INJECTIONS WERE EQUIVALENT TO 2 ML OF THE ORIGINAL VOLUME OF THE PLASMA PER 100 GM OF THE RECIPIENT'S BODY WEIGHT AVERAGE COUNTS OF 6 RATS RECEIVING EACH OF THESE MATERIALS

Status of Polycythemia Vera in Plasma Donors	*Determination*	*Baseline*	*1 Week*	*2 Weeks**	*3 Weeks*	*4 Weeks*
Active[1]	Plts	676	847	950	—	—
	WBC	14 3	14 8	13 5	—	—
Active[2]	Plts	675	900	932	731	680
	WBC	12 0	13 6	13 4	13 7	13 1
Active[3]	Plts	714	984	900	702	695
	WBC	10 8	12 6	13 0	11 3	10 0
Therapeutic Remission[4]	Plts	751	1 015	982	831	628
	WBC	12 2	11 3	13 3	14 5	13 8
Therapeutic Remission[5]	Plts	655	1 188	940	631	589
	WBC	14 5	12 3	12 9	13 0	12 8
Therapeutic Remission[6]	Plts	659	938	956	702	695
	WBC	10 3	13 6	13 3	11 3	10 0
Other Test Materials						
Normal Human Plasma Extract[7]	Plts	699	793	810	763	694
	WBC	12 7	12 3	13 2	10 2	10 2
Ringer's Solution[8]	Plts	632	600	646	619	674
	WBC	12 7	13 7	13 0	13 4	12 3

*Injections discontinued

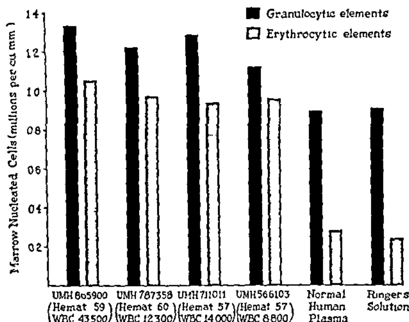


FIGURE 29 Erythrocytic and granulocytic hyperplasia at the end of a two week injection period in the marrows of the rats given the "polycythemic plasma extracts which induced the thrombocytosis and leukocytosis depicted in Figure 28 Average counts of two animals in each group The hematocrits and leukocyte counts of the plasma donors are shown in parentheses (From Linman J W Bethell F H and Long M J Factors controlling hemopoiesis Experimental observations on their role in polycythemia vera *Ann Int Med* 51 1003 1018 1959 Reprinted by permission)

themic plasma extracts Megakaryocytes were also increased Similar studies on the plasmas from four other patients with active polycythemia vera and one in therapeutic remission have corroborated the myelopoietic stimulatory activity of "polycythemic plasmas (Tables XVI and XVII) The recipients of a normal human plasma extract again manifested slight elevations in their thrombocyte counts without erythrocyte reticulocyte or leukocyte increases

The magnitude and uniformity of the platelet and leukocyte increases in these recipients of polycythemic plasma extracts

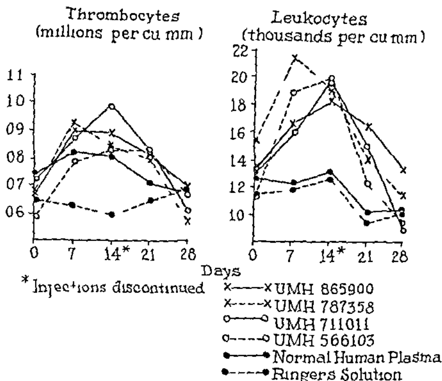


FIGURE 28 Thrombocytosis and leukocytosis in normal rats injected with the boiled extracts of plasmas from four patients with active polycythemia vera Daily injections were equivalent to four ml of the original plasma per 100 gm of the recipients body weights Counts in those animals not killed for marrow studies returned promptly to baseline levels after the injections were stopped Average counts of four animals receiving each of the above described test materials The hematocrits and leukocyte counts of the donors are shown in Figure 29 Each of these patients had moderate to marked thrombocytosis (From Linman J W Bethell F H and Long M J Factors controlling hemopoiesis Experimental observations on their role in polycythemia vera *Ann Int Med* 51 1003 1018 1959 Reprinted by permission)

the significance of this finding as do the marrow nucleated cell counts At the end of the treatment period half of the animals in each group were killed and their femoral marrows examined As shown in Figure 29 both erythrocytic and granulocytic hyper plasia were demonstrated in the rats injected with the "polycy

TABLE XVI

LEUKOCYTOSIS AND THROMBOCYTOSIS IN NORMAL RATS INJECTED WITH BOILED EXTRACTS OF PLASMAS FROM PATIENTS WITH POLYCYTHEMIA VERA AVERAGE COUNTS OF 4 RATS IN EACH GROUP GIVEN THESE MATERIALS AND OF 8 RECEIVING THE NORMAL PLASMA EXTRACT AND RINGERS SOLUTION (From Linman J W Bethell F H and Long M J Factors controlling hemopoiesis Experimental observations on their role in polycythemia vera *Ann Int Med* *51* 1003 1018 1959 Reprinted by permission)

Test Materials	*Daily Dose (ml / 100gm)*	*Leukocytes (x 10³) and Thrombocytes (x 10⁶) per cu mm*					
		De termi nation	*Base line*	*1 Week*	*2 Weeks**	*3 Weeks*	*4 Week*
UMH 852687 Hemat 55 WBC 13 050 Plts Normal	4	WBC	14 1	16 2	20 4	12 8	—
		Plts	500	864	995	745	610
UMH 514267 Hemat 52 WBC 10 000 Plts Increased	3	WBC	14 7	19 8	22 6	11 5	—
		Plts	604	852	893	647	661
UMH 811765 Hemat 52 WBC 14 500 Plts Increased	4	WBC	13 4	15 3	17 5	13 5	—
		Plts	6[illegible]2	929	998	746	701
UMH 707644 Hemat 47 WBC 15 250 Plts Increased	3	WBC	14 7	16 6	18 4	12 8	—
		Plts	5[illegible]8	856	972	745	694
UMH 835356 *Hemat 53* WBC 8 700 Plts Normal (3 months after Rx with P^{32})	3	WBC	11 2	19 1	26 2	14 0	—
		Plts	705	1 075	864	718	725
Normal Human Plasma	4	WBC	12 7	11 5	12 9	10 9	—
		Plts	718	784	805	718	709
Ringer s Solution	4	WBC	12 7	12 0	11 8	10 3	—
		Plts	699	700	667	705	706

*Injections Discontinued

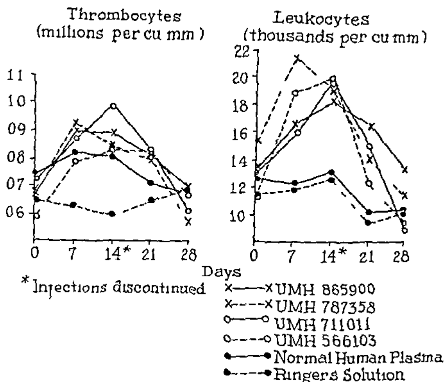


FIGURE 28 Thrombocytosis and leukocytosis in normal rats injected with the boiled extracts of plasmas from four patients with active polycythemia vera Daily injections were equivalent to four ml of the original plasma per 100 gm of the recipients body weights Counts in those animals not killed for marrow studies returned promptly to baseline levels after the injections were stopped Average counts of four animals receiving each of the above described test materials The hematocrits and leukocyte counts of the donors are shown in Figure 29 Each of these patients had moderate to marked thrombocytosis (From Linman J W Bethell F H and Long M J Factors controlling hemopoiesis Experimental observations on their role in polycythemia vera *Ann Int Med* 51 1003 1018 1959 Reprinted by permission)

the significance of this finding as do the marrow nucleated cell counts At the end of the treatment period half of the animals in each group were killed and their femoral marrows examined As shown in Figure 29 both erythrocytic and granulocytic hyper plasia were demonstrated in the rats injected with the polycy

However the thrombocytic and leukocytic stimuli in polycythemic plasmas have not been dissociated from the thermostable ether soluble plasma erythropoietic factor which has been studied The fact that a single compound batyl alcohol has been shown to affect the rate of production of all myeloid elements also rules against this possibility

Confirmation of the above hypothesis would clarify the pathogenesis of the proliferation of myeloid elements *en masse* in certain other conditions such as acute hemorrhage and hemolysis As previously discussed (see page 114) the failure to observe thrombocytosis and leukocytosis consistently in experimental animals and human subjects with myeloid erythrocytic hyperplasia secondary to enhanced plasma erythropoietic factor activity does not necessarily detract from this theory The findings in recipients of polycythemic plasma are in agreement with the relationship between dose and type of response suggested by the studies on the hemopoietic effects of batyl alcohol Larger amounts of these plasma extracts failed to induce greater erythrocytosis or thrombocytosis but did evoke leukocytosis in addition to the erythrocyte and thrombocyte increases This observation suggests reasonably but without proof that selective and preferential utilization of the active agent by erythrocytic precursors may occur Although the final response is undoubtedly governed by many variables it may be determined at least in part by the number of specific hemic elements present in the marrow at the time the stimulus is applied Since the total generation and intramitotic times of erythrocytic precursors are apparently fixed only a limited number of cellular divisions can take place from the time a cell enters the erythrocytic series until it has matured to a stage no longer capable of undergoing mitosis In the absence of a comparable increase in the number of erythrocytic precursors derived from the primitive reticulum cells and available to selectively utilize the mitotic stimulant the latter might then affect granulocytic precursors

The speculative nature of the above hypothesis is evident However several observations are seemingly in accord with this possibility 1) Plateaus are evident in the erythrocyte reticulocyte and marrow nucleated red cell counts in rats injected for

the rapid return to normal hemic equilibrium after the injections were stopped and the marrow findings establish the presence of thrombopoietic and granulopoietic activity in these plasmas from patients with polycythemia vera. However, varying degrees of sensitivity or responsiveness of the different myeloid elements to the stimulus contained in these materials exists, at least in the normal rat. Larger doses are needed of most but not all such plasmas in order to induce leukocytosis in recipient animals, whereas smaller amounts consistently exert demonstrable erythropoietic and thrombopoietic effects.

We have observed leukocytosis in rats given the thermostable fractions of plasmas from three patients with active polycythemia vera in daily doses equivalent to only two per cent of their body weight, but our experiences to date indicate that one and one half to two times this amount are usually required to elicit this response. In this connection it is of interest that the leukocyte increases in other rats given twice the quantity of the above plasma extracts were numerically comparable. It is also worthy of note that the smallest daily dosage employed in these experiments, i.e., the equivalent of 2 ml of the original plasma per 100 Gm of the recipient's body weight, apparently imparted the maximal stimulus to erythropoiesis and thrombopoiesis. The increases in the erythrocyte, thrombocyte and marrow nucleated red cell counts in the rats receiving the polycythemic plasma extracts were comparable in all groups irrespective of the size of the daily injection. Therefore it was not possible to quantitate the activity in these plasmas or relate either the degree or type of response in the recipient to the magnitude of the erythrocytosis, thrombocytosis or leukocytosis in the patients. It may be concluded, however, that the presence of active disease in the donor is not a prerequisite for the *in vitro* demonstration of plasma myelopoietic stimulatory activity.

The thrombocytosis and leukocytosis promoting properties of plasmas from patients with polycythemia vera accord strong support to the thesis that humoral factors are responsible for the thrombocyte and leukocyte increases associated with this disorder in addition to the accelerated rate of erythropoiesis. Thus all of the hematologic manifestations of polycythemia vera are the

centrates of normal human plasma contain demonstrable erythropoietic activity

The existence of a humoral hemopoietic regulatory mechanism demands more experimental confirmation than is currently available but it is tentatively proposed on the basis of data now at hand that the thermostable ether soluble factor may affect although not to the exclusion of other mechanisms the formation of all hemic elements However the entire subject needs further study before definite conclusions may be reached

In summary experimental observations indicate that the plasma erythropoietic factors play an important role in human erythropoiesis They are apparently responsible for the maintenance of the normal erythroid steady state and for the increased erythropoiesis which follows hemorrhage or hemolysis Augmented plasma erythropoietic activity is also demonstrable in patients with anemias due to well established marrow abnormalities or deficiency states However the latter preclude a normal marrow response to the humoral stimulus Although it has not yet been proved impaired production or defective utilization of the plasma factors may be of pathogenetic importance in some currently obscure anemias The humoral agents also appear to constitute the physiologic corrective mechanism which attempts to maintain an adequate supply of oxygen for the tissues in the face of decreased arterial oxygen saturation regardless of cause The inability to compensate for severe arterial hypoxemia eventuates in the production of an unphysiologic polycythemia

The presence of enhanced erythropoietic factor activity in the plasma of patients with polycythemia vera which occurs in the absence of hypoxia and persists after treatment with a myelosuppressive agent supports the thesis that an imbalance or derangement in the humoral regulatory mechanism is of etiologic significance in this disorder The thermostable or ether soluble fractions of plasmas from patients with polycythemia vera also induce thrombocytosis and leukocytosis in recipient rats These phenomena lend credence to the theory that all aspects of hemopoiesis may be under humoral control It is suggested that a single agent or activator inhibitor complex may affect the formation of all hemic elements and may explain the thrombocytosis and leu

TABLE XVII

MYELOID ERYTHROCYTIC AND GRANULOCYTIC HYPERPLASIA IN NORMAL RATS INJECTED WITH BOILED EXTRACTS OF PLASMAS FROM PATIENTS WITH POLYCYTHEMIA VERA (TABLE XVI) AVERAGE COUNTS OF 2 ANIMALS RECEIVING EACH OF THESE MATERIALS AND OF 4 GIVEN THE NORMAL PLASMA EXTRACT AND RINGER'S SOLUTION (From Linman J W Bethell F H and Long M J Factors controlling hemopoiesis Experimental observations on their role in polycythemia vera *Ann Int Med* 51 1003 1018 1959 Reprinted by permission)

Test Materials	Marrow Nucleated Cells per cu mm	
	Total Granulocytic	Total Erythrocytic
UMH 8,2687	1 300 228	1 011 585
UMH 514267	1 151 257	960 024
UMH 811765	1 286 823	989 561
UMH 707644	1 118 124	843 744
UMH 835356	1 114 934	935 450
Normal Human Plasma	948 427	291 229
Ringer s Solution	952 658	256 185

apparent result of enhanced plasma factor activity The stimulus to this derangement in the humoral regulatory mechanism remains unknown

The thrombopoietic and granulopoietic activity contained in the thermostable or other soluble fractions of plasmas from patients with polycythemia vera also provides direct support for the theory that all aspects of hemopoiesis are subject to humoral regulatory control and that a single substance or activator inhibitor complex may influence the formation of all hemic elements The possible presence of multiple humoral factors each affecting the rate of proliferation of a single cell type cannot be excluded

BIBLIOGRAPHY

1 Price Jones C *Red Blood Cell Diameters* London Oxford Medical Publications 1933

2 Maximow A A Relation of blood cells to connective tissue and endothelium *Physiol Rev* *4* 533-563 1924

3 Michels N A Erythropoiesis A critical review of the literature *Folia haemat* *45* 75 128 1931

4 Whitby L E H and Britton C J C *Disorders of the Blood* 7th Ed New York Grune and Stratton 1953

5 Wintrobe M M *Clinical Hematology* 4th Ed Philadelphia Lea and Febiger 1956

6 Granick S The chemistry and functioning of the mammalian erythrocyte *Blood* *4* 404 441 1949

7 Prankerd T A J The metabolism of the human erythrocyte A review *Brit J Haemat* *1* 131 145 1955

8 Condensation of the first two reports of the committee for clarification of the nomenclature of cells and diseases of the blood and blood forming organs The terms and definitions for the cells of the leukocytic thrombocytic and erythrocytic series *Blood* *4* 89 96 1949

9 Cartwright G E Dietary factors in erythropoiesis *Blood* *2* 111 153 and 256 298 1947 Two parts

10 Conference on Hemoglobin Publication 557 Washington D C National Academy of Sciences National Research Council 1958

11 Gordon A S and Charipper H A The endocrine system and hemopoiesis *Ann New York Acad Sc* *48* 615 637 1947

12 Daughaday W H Williams R H and Daland G A The effects of endocrinopathies on the blood *Blood* *3* 1342 1366 1948

13 Gordon A S Endocrine influences upon the formed elements of blood and blood forming organs *Recent Prog Hormone Res* *10* 339-394 1954

14 Van Dyke D C Contopoulos A N Williams B S Simpson M E Lawrence J H and Evans H M Hormonal factors influencing erythropoiesis *Acta haemat* *11* 203 222 1954

prolonged periods with boiled extracts of anemic plasmas (see page 87) 2) Activity attributable to the thermostable ether soluble erythropoietic factor is apparently more marked in patients with polycythemia vera than is that of the humoral factor which augments hemoglobin synthesis 3) Leukocytosis is not present in patients with secondary polycythemia where arterial hypoxemia ensures through its enhancing effect on the relatively thermolabile plasma erythropoietic factor an increased rate of reticulum cell erythrocytic differentiation 4) The thrombocytosis and leukocytosis associated with acute hemorrhage occur promptly and are soon replaced by evidence of active red cell regeneration i e polychromasia reticulocytosis and nucleated red cells in the circulating blood

An alternative explanation might be that granulocytic precursors are not affected until a certain level of humoral factor activity is reached The persistence of leukocytosis in patients with some types of hemolytic disease supports this possibility The problem is further complicated by preliminary observations which indicate that microcytes with decreased osmotic resistance may be present in some patients with secondary polycythemia in the absence of thrombocytosis or leukocytosis More information is needed about the elaboration or activation utilization inactivation and excretion of the plasma factors In addition the mechanisms responsible for the differentiation of the pluripotential reticulum cells into granulocytic and megakaryocytic precursors are unknown This aspect of blood cell formation represents a field of hematologic research which is virtually unexplored and must be subjected to intensive study before certain of the problems relating to the control of hemopoiesis may be solved

The significance if any of the minimal thrombopoietic effect of normal human plasma (Fig 28 Tables XV and XVI) is conjectural However this finding is compatible with the observations of Schulman and his associates[370] on a child with chronic thrombocytopenia Normal plasma has repeatedly but temporarily restored the platelet counts to normal in this child This finding suggests that a plasma thrombopoietic factor is present in normal individuals In this connection it is of interest that con

28 Boenheim F Schwimmer D and McGavack T H The combination of hyperthyroidism and pernicious anemia Report of a case with a review of the literature *Ann Int Med* 23 869-882 1946
29 Meyer O O Stewart G E Thewlis E W and Rusch H P The hypophysis and hematopoiesis *Folia haemat* 57 99 109 1937
30 Meyer O O Thewlis E W and Rusch H P The hypophysis and hemopoiesis *Endocrinology* 27 932 944 1940
31 Gordon A S Kadow P C Finkelstein G and Charipper H A The thyroid and blood regeneration in the rat *Am J M Sc* 212 385-394 1946
32 Ferrari R Influenza della milza sulle variazioni determinate dalla castrazione e dal trapianto di eonadi eterosessuali nel numero degli eritrociti e dei leucociti nella quantita di emo globina e nella formula leucocitaria *Haematologica* 11 425 428 1930
33 Steinglass P Gordon A S and Charipper H A Effect of castration and sex hormones on blood of the rat *Proc Soc Exper Biol & Med* 48 169 177 1941
34 McCullagh E P and Jones R Effect of androgens on the blood count of men *J Clin Endocrinol* 2 243 251 1942
35 Vollmer E P and Gordon A S Sex hormones and blood cells *Endocrinology* 29 828 837 1941
36 Finkelstein G Gordon A S and Charipper H A The effect of sex hormones on the anemia induced by hemorrhage in the rat *Endocrinology* 35 267 277 1944
37 Crafts R C The effect of endocrines on the formed elements of the blood II The effect of estrogens in the dog and mon key *Endocrinology* 29 606-618 1941
38 Kennedy B J and Nathanson I T Effects of intensive sex steroid hormone therapy in advanced breast cancer *J A M A* 152 1135 1141 1953
39 Kennedy B J and Gilbertsen A S Increased erythropoiesis induced by androgenic hormone therapy *New England J Med* 256 719 726 1957
40 Piliero S J Influence of hypoxic stimuli upon blood formation in endocrine deficient rats *Ann New York Acad Sc* 77 518 542 1959

locytosis associated with polycythemia vera and certain other conditions such as acute blood loss

The problem of the physiologic and pathophysiologic control of hemopoiesis is complex and has not yet been solved Recent studies have greatly advanced our knowledge of the remarkable homeostatic mechanism which governs erythropoiesis but there is still much to be learned Investigative approaches to the multiple facets of this problem are now underway in many laboratories Although many questions remain unanswered existent experimental observations are such that the primary importance of humoral regulatory mechanisms can no longer be denied Continued study of the humoral control of hemopoiesis should make possible the practical application of the basic information obtained toward the elucidation of certain poorly understood hematologic disorders and responses in man

56 Van Dyke D C Garcia J F Simpson M E Huff R L Contopoulos A N and Evans H M Maintenance of circulating red cell volume in rats after removal of the posterior and intermediate lobes of the pituitary *Blood* 7 1017 1019 1952
57 Silberglert A Effect of hypophysectomy on blood regeneration in the adult female rat *Proc Soc Exper Biol & Med* 79 170 173 1952
58 Baker B L Pliske E C Kent J F McGinty J D Van Dyke J E and Bethell F H Response of the blood corpuscles in hypophysectomized rats to irradiation with x ray *Endocrinology* 57 472-480 1955
59 Crafts R C The effects of cobalt liver extract and vitamin B_1 on the anemia induced by hypophysectomy in adult female rats *Blood* 7 863 873 1952
60 Garcia J F Van Dyke D C and Berlin N I Correction of anemia following hypophysectomy by administration of cobalt *Proc Soc Exper Biol & Med* 80 472-474 1952
61 Stewart G E Greep R O and Meyer O O Effect of reduced oxygen tension upon formed elements of blood of hypophysectomized animals *Proc Soc Exper Biol & Med* 33 112 114 1935
62 Feigin W M and Gordon A S Influence of hypophysectomy on the hemopoietic response of rats to lowered barometric pressures *Endocrinology* 47 364 369 1950
63 Crafts R C The effects of iron copper and thyroxine on the anemia induced by hypophysectomy in the adult female rat *Am J Anat* 79 267 291 1946
64 Garcia J F Van Dyke D C Huff R L Elmlinger P J and Oda J M Increase in circulating red cell volume of normal and hypophysectomized rats after treatment with ACTH *Proc Soc Exper Biol & Med* 76 707 709 1951
65 Crafts R C The prevention of anemia in hypophysectomized adult female rats with combined thyroxine and cortisone therapy *Endocrinology* 54 84 92 1954
66 Gemzell C A and Sjostrand T Effect of hypophysectomy ACTH and growth hormone on total amount of haemoglobin and blood volume in male rats *Acta endocrinol* 16 6 12 1954
67 Meineke H A and Crafts R C The effect of growth hormone on hematopoiesis in hypophysectomized adult female rats *Endocrinology* 59 444-453 1956

15 Crafts R C Relationships of hormones to the utilization of essential nutrients in erythropoiesis *Am J Clin Nutrition* 3 52 55 1955
16 Gordon A S Tornetta F J D Angelo S A and Charipper H A Effects of low atmospheric pressures on the activity of the thyroid reproduction system and anterior lobe of the pituitary in the rat *Endocrinology* 33 366 383 1943
17 Stern B and Altschule M D Hematological studies in hypothyroidism following total thyroidectomy *J Clin Investigation* 15 633 641 1936
18 Sharpe J C and Bisgard J D The thyroid gland and hematopoiesis II The effect of thyroid extract liver extract and iron on the anemia of myxedema *J Lab & Clin Med* 23 219 230 1937
19 Crafts R C The effects of endocrines on the formed elements of the blood I The effects of hypophysectomy thyroidectomy and adrenalectomy on the blood of the female rat *Endocrinology* 29 596 605 1941
20 Bomford R Anaemia in myxoedema and the role of the thyroid gland in erythropoiesis *Quart J Med* 7 495 536 1938
21 Jacobson L O Goldwasser E Fried W and Plzak L Control of erythropoiesis *Bull Alumni Assn Univ of Chicago School of Med* 14 1 4 1957
22 Jacobson L O and Goldwasser E The dynamic equilibrium of erythropoiesis Brookhaven Symposia in Biology No 10 Homeostatic Mechanisms 1958 pp 110 131
23 Latta J S and Benner M C The hemopoietic disturbances induced in the albino rat by administration of thyroxin *Am J Anat* 54 115 141 1934
24 Larizzo P and Chirico G La posizione della tiroide nella regolazione dell emopoiesi *Haematologica* 38 771 846 1954
25 Hoskins R G and Jellinek E M Studies on thyroid medication I Some conditions determining the haematopoietic effects *Endocrinology* 16 455 486 1932
26 Wilson T E The thyroid gland and haemopoiesis *M J Australia* 1 261 269 1944
27 Bistrom O On the morphology of blood and bone marrow in thyrotoxicosis *Acta chir scandinav* 94 1 188 1946 (Supp 114)

79 Van Dyke D C Simpson M E Contopoulos A N and Evans H M The separate existence of the pituitary erythropoietic hormone *Blood* 12 539 548 1957

80 Van Dyke D C The pituitary erythropoietic factor *Ann New York Acad Sc* 77 534-550 1959

81 Grinzell C A Van Dyke D C Tobias C A, and Evans H M Increase in the formation and secretion of ACTH following adrenalectomy *Endocrinology* 49 325 336 1951

82 Birkhill F R Maloney M A and Levenson S M Effect of transfusion polycythemia upon bone marrow activity and erythrocyte survival in man *Blood* 6 1021 1033 1951

83 Gurney C W and Pan C Studies on erythropoiesis IX Mechanism of decreased erythropoiesis in experimental polycythemia *Proc Soc Exper Biol & Med* 98 789 793 1958

84 Reinhard E H Moore C V Dubach R and Wade L J Depressant effects of high concentrations of inspired oxygen on erythrocytogenesis Observations on patients with sickle cell anemia with a description of the observed toxic manifestations of oxygen *J Clin Investigation* 23 682 698 1944

85 Tinsley J C Jr Moore C V Dubach R Minnich V and Grinstein M The role of oxygen in the regulation of erythropoiesis Depression of the rate of delivery of new red blood cells to the blood by high concentrations of inspired oxygen *J Clin Investigation* 28 1544 1564 1949

86 Evans E S Contopoulos A N and Simpson M E Hormonal factors influencing calorigenesis *Endocrinology* 60 403-419 1957

87 Crafts R C and Meineke H A Decreased oxygen need as a factor in anemia of hypophysectomized animals *Proc Soc Exper Biol & Med* 95 127 131 1957

88 Sunahara F A and Beck L Cardiovascular effects of acutely produced anemia in the normal dog *Am J Physiol* 176 139 142 1954

89 Fowler N O Franch R H and Bloom W L Hemodynamic effects of anemia with and without plasma volume expansion *Circulation Res* 4 319-324 1956

90 Grant, W C and Root W S Fundamental stimulus for erythropoiesis *Physiol Rev* 32 449 498 1952

91 Cruz W O and Robscheit Robbins F S Relationship between the spleen and the morphologic picture of blood regeneration *Am J M Sc* 203 28 34 1942

41 Gordon A S and Piliero S J The adrenal gland and hemopoiesis in the rat *Federation Proc* 9 49 50 1950

42 Piliero S J Landau D and Gordon A S The adrenal gland and hemopoiesis *Science* 112 559 560 1950

43 Gordon A S Piliero S J and Landau D The relation of the adrenal to blood formation in the rat *Endocrinology* 49 497 511 1951

44 Yoffey J M Ancill R J Holt J A G Owensmith B and Herdan G A quantitative study of the effects of compound E compound F and compound A upon the bone marrow of the guinea pig *J Anat* 88 115 130 1954

45 Fruhman G J and Gordon A S A quantitative study of adrenal influences upon the cellular elements of bone marrow *Endocrinology* 57 711 718 1955

46 Larizza P Chirico G and Notario A Interfereze surrenaliche nella regolazione dell emopoiesi *Haematologica* 39 37 147 1955

47 Fruhman G J and Gordon A S Effects of growth hormone and cortisol upon hemopoiesis *Acta haemat* 15 249 262 1956

48 Fisher J W Increase in circulating red cell volume of normal rats after treatment with hydrocortisone or corticosterone *Proc Soc Exper Biol & Med* 97 502 505 1958

49 Baez Villaseñor J Rath C E and Finch C A The blood picture in Addison s disease *Blood* 3 769 773 1948

50 Piliero S J The effects of low pressure on hemopoiesis in the adrenalectomized rat *Endocrinology* 57 676 682 1955

51 Nelson R Comparison of erythropoietic response in normal and adrenalectomized rats following hemorrhage *Federation Proc* 10 97 1951

52 Fisher J W Effects of adrenalectomy on cobalt polycythemia in the rat *Federation Proc* 16 296 1957

53 Berlin N I Van Dyke D C Siri W E and Williams C P The effect of hypophysectomy on the total circulating red cell volume of the rat *Endocrinology* 47 429-435 1950

54 Crafts R C and Meineke H A Influence of the pituitary on hemopoiesis *Am J Clin Nutrition* 5 453 460 1957

55 Crafts R C and Meineke H A The anemia of hypophysectomized animals *Ann New York Acad Sc* 77 501 517 1959

79 Van Dyke D C Simpson M E Contopoulos A N and Evans H M The separate existence of the pituitary erythropoietic hormone *Blood* 12 539 548 1957
80 Van Dyke D C The pituitary erythropoietic factor *Ann New York Acad Sc* 77 534-550 1959
81 Gemzell C A Van Dyke D C Tobias C A and Evans H M Increase in the formation and secretion of ACTH following adrenalectomy *Endocrinology* 49 325 336 1951
82 Birkhill F R Maloney M A and Levenson S M Effect of transfusion polycythemia upon bone marrow activity and erythrocyte survival in man *Blood* 6 1021 1033 1951
83 Gurney C W and Pan C Studies on erythropoiesis IX Mechanism of decreased erythropoiesis in experimental polycythemia *Proc Soc Exper Biol & Med* 98 789 793 1958
84 Reinhard E H Moore C V Dubach R and Wade L J Depressant effects of high concentrations of inspired oxygen on erythrocytogenesis Observations on patients with sickle cell anemia with a description of the observed toxic manifestations of oxygen *J Clin Investigation* 23 682 698 1944
85 Tinsley J C Jr Moore C V Dubach R Minnich V and Grinstein M The role of oxygen in the regulation of erythropoiesis Depression of the rate of delivery of new red blood cells to the blood by high concentrations of inspired oxygen *J Clin Investigation* 28 1544 1564 1949
86 Evans E S Contopoulos A N and Simpson M E Hormonal factors influencing calorigenesis *Endocrinology* 60 403-419 1957
87 Crafts R C and Meineke H A Decreased oxygen need as a factor in anemia of hypophysectomized animals *Proc Soc Exper Biol & Med* 95 127 131 1957
88 Sunahara F A and Beck L Cardiovascular effects of acutely produced anemia in the normal dog *Am J Physiol* 176 139 142 1954
89 Fowler N O Franch R H and Bloom W L Hemodynamic effects of anemia with and without plasma volume expansion *Circulation Res* 4 319-324 1956
90 Grant W C and Root W S Fundamental stimulus for erythropoiesis *Physiol Rev* 32 449-498 1952
91 Cruz W O and Robscheit Robbins F S Relationship between the spleen and the morphologic picture of blood regeneration *Am J M Sc* 203 28-34 1942

68 Meineke H A and Crafts R C Effect of combined thyroxin cortisone growth hormone therapy on hematopoiesis in hypophysectomized rats *Proc Soc Exper Biol & Med* *96* 74 79 1957

69 Meineke H A and Crafts R C Further observations on the effect of cortisone and thyroxin on the blood picture of hypophysectomized rats *Blood* *12* 11 23 1957

70 Crafts R C and Walker B S The effects of hypophysectomy on serum and storage iron in adult female rats *Endocrinology* *41* 340 346 1947

71 Marinone G and Meduri D Influences hypophysaires sur le metabolisme du fer *Rev hemat* *12* 169 191 1957

72 Meineke H A and Crafts R C A study of the anemia induced by hypophysectomy and that induced by combined thyroidectomy and adrenalectomy in Wistar Long Evans and Sprague Dawley rats *Anat Rec* *124* 47 66 1956

73 Van Dyke D C Simpson M E Garcia J F and Evans H M Inability of growth hormone to prevent the anemia which follows hypophysectomy *Proc Soc Exper Biol & Med* *81* 574 576 1952

74 Flaks J Himmel I and Zotnik A La polyglobulie provoquée par les extraits de lobe anterieur d hypophyse prouve l existence d une hormone hemopoietique *Presse med* *46* 1506 1509 1938

75 Contopoulos A N Van Dyke D C Simpson M E Garcia J F Huff R L Williams B S and Evans H M Increase in circulating red cell volume after oral administration of pituitary anterior lobe *Blood* *8* 131 139 1953

76 Contopoulos A N Ellis S Simpson M E Lawrence J H and Evans H M Production of polycythemia in hypophysectomized rats by the pituitary erythropoietic factor *Endocrinology* *55* 808 812 1954

77 Contopoulos A N Van Dyke D C Ellis S Simpson M E Lawrence J H and Evans H M Evidence for a pituitary erythropoietic factor *Gaz med Portuguesa* *7* 99 104 1954

78 Contopoulos A N Van Dyke D C Ellis S Simpson M E Lawrence J H and Evans H M Prevention of neonatal anemia in the rat by the pituitary erythropoietic factor *Blood* *10* 115 119 1955

108 Gerhardt D Die entstehung und behandlung der sekundaren anamien *Verhandl deutsch Kong innere Med* 27 109 156 1910

109 Boycott A E Douglas C G and Price Jones C Haemopoietine *J Path & Bact* 15 116 118 1911

110 Gibelli C Über den wert des serums anamisch gemachten tiere bei der regeneration des blutes *Arch exp Path Pharmakol* 65 284-302 1911

111 Muller P Th Über die wirkung des blutserums anamischer tiere *Arch Hyg* 75 290-320 1912

112 Morawitz P Einige neuere anschauungen uber blutregeneration *Ergebn inn Med Kinderh* 11 277-323 1913

113 Forster J Luftverdunnung und blut regeneration du ch hamopoietine *Biochem Ztschr* 145 309-317 1924

114 Gordon A S and Dubin M On the alleged presence of hemopoietine in the blood serum of rabbits either rendered anemic or subjected to low pressures *Am J Physiol* 107 704 708 1934

115 Feenders H Über die hamatopoetiscke wirkung des blutserums bei sekundarer anamie *Frankfurt Ztschr Path* 49 411-417 1936

116 Tei Y Production of hematopoietic substances in serum J Chosen M A 28 No 9 179 1938

117 Bonsdorff E and Jalavisto E A humoral mechanism in anoxic erythrocytosis *Acta physiol scandinav* 16 150 170 1948 (supp 53)

118 Reissmann K R Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia *Blood* 5 372 380 1950

119 Erslev A Humoral regulation of red cell production *Blood* 8 349 357 1953

120 Borsook H Graybiel A Keighley G and Windsor E Polycythemic response in normal adult rats to a nonprotein plasma extract from anemic rabbits *Blood* 9 734 742 1954

121 Gordon A S Piliero S J Kleinberg W and Freedman H H A plasma extract with erythropoietic activity *Proc Soc Exper Biol & Med* 86 255 258 1954

122 Sacks M S Editorial Erythropoietin *Ann Int Med* 48 207 212 1958

123 Gordon A S Hemopoietine *Physiol Rev* 39 1 40 1959

92 Dameshek W and Estren S *The Spleen and Hypersplenism* New York Grune and Stratton 1947

93 Sturgis C C *Hypersplenism A Clinical Evaluation* Springfield Charles C Thomas 1953

94 Hayhoe F G J and Whitby L Splenic function A study of rationale and results of splenectomy in blood disorders *Quart J Med* *24* 365 391 1955

95 Crosby W H Normal functions of the spleen relative to red blood cells A review *Blood* *14* 399 408 1959

96 Dameshek W and Schwartz R Hemolytic mechanisms *Ann New York Acad Sc* *77* 589 614 1959

97 Bert P *La Pression Barométrique* Paris G Masson 1878

98 Bert P Sur le richesse en hemoglobine du sang des animaux vivant sur les hauts lieux *Compt rend Acad sc* *94* 805 807 1882

99 Viault F Sur l augmentation considerable du nombre des globules rouges dans le sang chez les habitants des hauts plateaux de l Amerique du Sud *Compt rend Acad sc* *111* 917 1890

100 Muntz A De l enrichissement du sang en hemoglobine suivant les conditions d existence *Compt rend Acad sc* *112* 298 301 1891

101 Miescher F Bemerkungen uber eine verbesserte form der mischpipette und ihren einfluss auf die genauigkeit der blut korperzahlung *Cor Bl f schweiz Aerzte* *23* 830 832 1893

102 Grant W C and Root W S The relation of O in bone marrow blood to post hemorrhagic erythropoiesis *Am J Physiol* *150* 618 627 1947

103 Grant W C Oxygen saturation in bone marrow and in arterial and venous blood during prolonged hemorrhagic erythropoiesis *Am J Physiol* *153* 521 528 1948

104 Magnussen J D The influence of oxygen tension on the production of erythrocytes *in vitro* *Acta pharmacol et toxicol* *5* 153 163 1949

105 Thomas E D *In vitro* studies of erythropoiesis II The effect of anoxia on heme synthesis *Blood* *10* 612 615 1955

106 Carnot P and Deflandre C Sur l activite hemopoietique du serum au cours de la regeneration du sang *Compt rend Acad sc* *143* 384-386 1906

107 Carnot P and Deflandre C Sur l activite hemopoietique des differents organes au cours de la regeneration du sang *Compt rend Acad sc* *143* 432 435 1906

137 Lulby A I Cooperman J M Herrero J M Gordon A S Piliero S J and Medici P T Clinical experiences with erythropoietin *Clin Res Proc 6* 194 1959

138 Plzak L F Fried W Jacobson L O and Bethard W F Demonstration of stimulation of erythropoiesis by plasma from anemic rats using Fe [59] *J Lab & Clin Med 46* 671 678 1955

139 Rambach W A Cooper J A D and Alt H L The nature of erythropoietin *Ann New York Acad Sc 77* 623 626 1959

140 Fried W Plzak L Jacobson L O and Goldwasser E Erythropoiesis II Assay of erythropoietin in hypophysectomized rats *Proc Soc Exper Biol & Med* 92 203 207 1956

141 Jacobson L O Plzak L Fried W and Goldwasser E Plasma factor(s) influencing red cell production *Nature 177* 1240 1956

142 Fried W Plzak L F Jacobson L O and Goldwasser E Studies on erythropoiesis III Factors controlling erythropoietin production *Proc Soc Exper Biol & Med 94* 237 241 1957

143 Mirand E A and Prentice T C A simple test for erythropoietin in deproteinized plasma extract *Proc Soc Exper Biol & Med 93* 473-475 1956

144 Marmone G Medori D Petronio L and Puricelli G Sopra l influenza dei fattori plasmatici sull attivita eritropoietica midollare *Hematologica 41* 941 962 1956

145 Fruhman G J and Gordon A S Influence of starvation upon the formed elements of blood and bone marrow of the rat *Anat Rec 122* 492 1955

146 Jacobson L O Goldwasser E Fried W and Plzak L Role of kidney in erythropoiesis *Nature 179* 633 634 1957

147 Jacobson L O Goldwasser E Fried W and Plzak L The role of the kidney in the control of erythropoiesis *Tr A Am Physicians 70* 305 317 1957

148 Stohlman F Jr and Brecher G Stimulation of erythropoiesis in sublethally irradiated rats by a plasma factor *Proc Soc Exper Biol & Med 91* 1-4 1956

149 Bethell F H Linman J W and Korst D R Erythropoietic activity of "anemic" and "polycythemic" plasmas *Tr A Am Physicians 70* 297-304 1957

124 Bethell F H and Meyers M C *Clinical Laboratory Diagnosis and Essentials of Hematology* 4th Ed Ann Arbor Cushing Malloy Inc 1954 pp 110 111

125 Seip M Reticulocyte studies *Acta med scandinav 146* 1 164 1953 (Supp 282)

126 Hodgson G Eskuche I Yudilevich, D Hernandez P and Tohá J Effect of plasma from bled and phenylhydrazine treated animals on plasma iron turnover A test for hemopoietine" *Proc Soc Exper Biol & Med 96* 826 829 1957

127 Erslev A J Lavietes P H and van Wagenen G Erythropoietic stimulation induced by anemic serum *Proc Soc Exper Biol & Med 83* 548 550 1953

128 Keighley G Borsook H and Graybiel A Production of increased circulating hemoglobin in mice *Science 124* 439 1956

129 Jacobson L O Goldwasser E Plzak L F and Fried W Studies on erythropoiesis IV Reticulocyte response of hypophysectomized and polycythemic rodents to erythropoietin *Proc Soc Exper Biol & Med 94* 243 249 1957

130 Mirand E A and Prentice T C Fe^{59} assessment of erythropoiesis in mice following injections of anemic rabbit plasma extracts *Proc Soc Exper Biol & Med 95* 164 167 1957

131 Lowy P H Keighley G Borsook H and Graybiel A On the erythropoietic principle in the blood of rabbits made severely anemic with phenylhydrazine *Blood 14* 262 273 1959

132 Bonsdorff E Erythrocytosis promoting activity in stagnant blood and in blood subjected to low atmospheric pressure *in vitro Ann med exper et biol Fenniae 31* 1 77 1953 (Supp 3)

133 Sandler O E Some experimental studies on the erythropoietic effect of yellow bone marrow extracts and batyl alcohol *Acta med scandinav 133* 1 72 1949 (Supp 225)

134 Oliva G Chiuni, F and Tramontana C On the humoral regulation of the normoerythropoiesis *Acta med scandinav 133* 27 30 1949

135 Gurney *C W Pierce M I Schrier S E Carson P E and* Jacobson L O The stimulatory effect of "anemic plasma in congenital hypoplastic anemia *J Lab & Clin Med 50* 821 1957

136 McCurdy P R Miskovsky E Laughlin T and McCuiston C F Studies of plasma erythropoietic factor in man *Clin Res Proc 6* 193 1958

164 Garcia J F Erythropoietic response to hypoxia as a function of age in the normal male rat *Am J Physiol* *190* 25-30 1957

165 Harris R S Herdan G Ancill R J and Yoffey J M A quantitative comparison of the nucleated cells in the right and left humeral bone marrow of the guinea pig *Blood* *9* 374 378 1954

166 Gordon A S Some aspects of hormonal influences upon the leukocytes *Ann New York Acad Sc* *59* 907 927 1955

167 Yoffey J M The quantitative study of the leukocytes *Ann New York Acad Sc* *59* 928 940 1955

168 Fand I and Gordon A S A quantitative study of the bone marrow in the guinea pig throughout life *J Morphol* *100* 473-507 1957

169 Hudson G Bone marrow volume in guinea pigs *J Anat* *92* 150 161 1958

170 Berlin N I Huff R L Van Dyke D C and Hennessy T G The blood volume of the adult rat as determined by Fe [59] and P[32] labelled red cells *Proc Soc Exper Biol & Med* *71* 176 178 1949

171 Linman J W and Bethell F H The plasma erythropoietic stimulating factor Observations on circulating erythrocytes and bone marrow of rats receiving protein free extracts of rabbit plasma *Blood* *11* 310-323 1956

172 Linman J W and Long M J Erythrocyte osmotic fragility of rats receiving the thermostable plasma erythropoietic factor *Blood* *13* 226 238 1958

173 Simmel H Die osmotische resistenz der erythrocyten *Deutsches Arch klin Med* *142* 252 265 1923

174 Rodnan G P Ebaugh F G Jr and Fox M R S The life span of the red blood cell and the red blood cell volume in the chicken pigeon and duck as estimated by the use of $Na_2Cr^{51}O_4$ *Blood* *12* 355 366 1957

175 Van Putten L M The life span of red cells in the rat and the mouse as determined by labeling with DFP[32] *in vivo* *Blood* *13* 789 794 1958

176 Jaffe E R Vanderhoff G A Lowy B A and London I M The effect of nucleosides on osmotic resistance of mammalian erythrocytes in relation to the age of the cells *Clin Res Proc* *5* 140 1957

150 Korst D R Bishop R C and Bethell F H A say of erythropoietic factor(s) using radioiron uptake in the nitrogen mustard treated rat *J Lab & Clin Med* 52 364 374 1958
151 Korst D R and Bethell F H Erythrocyte radioiron uptake in the rat and the effects of cobalt *J Lab & Clin Med* 52 356-363 1958
152 Matoth Y Biezunski N and Szabo G Effect of sera from patients with anoxia on the proliferative activity of human erythropoietic tissue *in vitro* *J Lab & Clin Med* 51 420 428 1958
153 Friederici L Über den nachweis des erythropoetins in der gewebekultur *Klin Wchnschr* 36 132 134 1958
154 Rosse W F and Gurney C W Studies on erythropoiesis X The use of bone marrow tissue culture in demonstrating erythropoietin *J Lab & Clin Med*, 53 446-456 1959
155 Reisner E H Jr Tissue culture of bone marrow *Ann New York Acad Sc* 77 487 500 1959
156 Schroeder L R Gurney C W and Wackman N Assay of erythropoietin in bone marrow suspensions *Nature* 181 1537 1538 1958
157 Korst D R Wilhelm J and Bethell F H Erythropoietin assay *J Lab & Clin Med* 58 850 1958
158 Linman J W Long M J Korst D R and Bethell F H Studies on the stimulation of hemopoiesis by batyl alcohol *J Lab & Clin Med* 54 335-343 1959
159 Jandl J H Inman J K Simmons R L and Allen D W Transfer of iron from serum iron binding protein to human reticulocytes *J Clin Investigation* 38 161 185 1959
160 Kuna S Gordon A S and Charipper H A Bone marrow function in isolated perfused hind limbs of rats *Federation Proc*, 16 76 1957
161 Gordon A S Dornfest B S Siegel C D and Gottlieb N Hemopoiesis in isolated perfused hind limbs of hypophysectomized rats *Federation Proc* 17 57 1958
162. Gordon A S Winkert J Dornfest B S and Siegel C D Studies on the actions and properties of the circulating erythropoietic stimulating factor *Ann New York Acad Sc* 77 650 676 1959
163 Rambach W A Alt H L and Cooper J A D The mode of action and nature of a heat stable plasma erythropoietic factor *Blood* 12 1101 1113 1957

188 Linman J W and Bethell F H The plasma erythropoietic stimulating factor in man Observations on patients with polycythemia vera and secondary polycythemia *J Lab & Clin Med* 49 113 127 1957

189 Linman J W and Bethell F H The effect of irradiation on the plasma erythropoietic stimulating factor *Blood* 12 123 129 1957

190 Gurney C W Goldwasser E and Pan C Studies on erythropoiesis VI Erythropoietin in human plasma *J Lab & Clin Med* 50 534-542 1957

191 Prentice T C and Mirand E A Studies of plasma erythropoietic factor in anemic human patients *Blood* 12 993 997 1957

192 Contopoulos A N McCombs R Lawrence J H and Simpson M E Erythropoietic activity in the plasma of patients with polycythemia vera and secondary polycythemia *Blood* 12 614 619 1957

193 Rambach W A Cooper J A D and Alt H L Purification of erythropoietin by ion exchange chromatography *Proc Soc Exper Biol & Med* 98 602 604 1958

194 Rambach W A Shaw R A Cooper J A D and Alt H L Acid hydrolysis of erythropoietin *Proc Soc Exper Biol & Med* 99 482-483 1958

195 Borsook H A discussion of humoral erythropoietic factors *Ann New York Acad Sc* 77 725 736 1959

196 Hodgson G Yudilevich D Perretta M Eskuche I and Toha J Effect of hemopoietin on iron metabolism in normal and starved animals Studies with Fe [59] *Ann New York Acad Sc* 77 703 709 1959

197 Mirand E A Prentice T C and Slaunwhite W R Current studies on the role of erythropoietin on erythropoiesis *Ann New York Acad Sc* 77 677 702 1959

198 Erslev A J Physiologic control of red cell production *Blood* 10 954 961 1955

199 Stohlman F Jr and Brecher G Humoral regulation of erythropoiesis IV Relative heat stability of erythropoietine *Proc Soc Exper Biol & Med* 95 797 800 1957

200 Gurney C W Jacobson L O and Goldwasser E The physiologic and clinical significance of erythropoietin *Ann Int Med* 49 363-370 1958

177 Marks P A A relationship between human erythrocyte aging *in vivo* and the activities of glucose 6 phosphate and 6 phosphogluconic dehydrogenases *J Clin Investigation* *36* 913 1957
178 Schultze M O Metallic elements and blood formation *Physiol Rev* *20* 37 67 1940
179 Linman J W Bethell F H and Tascott H Additional observations on the plasma erythropoietic stimulating factor Progress Report University of Michigan Project for the Study of the Biological Effects of Irradiation 1956 pp 72 78
180 Linman J W Bethell F H and Long M J Studies on the nature of the plasma erythropoietic factor(s) *J Lab & Clin Med* *51* 8 16 1958
181 Toha J Eskuche I Abarca F Salvatore F and Hodgson G Chemical properties of a plasma factor accelerating hemoglobin recovery in bled rabbits *Nature* *175* 167 168 1955
182 Gordon A S Piliero S J Tannenbaum M and Siegel C D Erythropoietic action of a plasma filtrate in hypophysectomized rats *Proc Soc Exper Biol & Med* *89* 246-248 1955
183 Gordon A S Piliero S J and Tannenbaum M Erythropoietic activity of blood and tissues of anemic rabbits *Am J Physiol* *181* 585 588 1955
184 Gordon A S Piliero S J Medici P T Siegel C D and Tannenbaum M Attempts to identify site of production of circulating erythropoietin *Proc Soc Exper Biol & Med* *92* 598 602 1956
185 Piliero S J Medici P T Pansky B Luhby A L and Gordon A S Erythropoietic stimulating effects of plasma extracts from anemic human subjects *Proc Soc Exper Biol & Med* *93* 302 305 1956
186 Jacobsen E M Davis A K and Alpen E L Relative effectiveness of phenylhydrazine treatment and hemorrhage in the production of an erythropoietic factor *Blood* *11* 937 945 1956
187 Prentice T C and Mirand E A Erythropoietic action of a plasma extract from animals with varying degrees of anemia in normal rats *Exper Med & Surg* *14* 226 238 1956

215 Linkenheimer W H Grant W C and Berger H Effect of anemic rabbit plasma on erythropoiesis in the rat *Federation Proc* *15* 121 1956

216 Van Dyke D C and Garcia J F The chemical characteristics of urinary erythropoietin *Clin Res Proc* *6* 44 1958

217 Winkert J Gordon A S Piliero S J and Medici P T Partial purification of urinary erythropoietin *Proc Soc Exper Biol & Med* *98* 351-354 1958

218 Hodgson G Perreta M Yudilevich D and Eskuche I Assay of hemopoietine in starved animals properties of urinary hemopoietine *Proc Soc Exper Biol & Med* *99* 137 142 1958

219 Medici P T Gordon A S Piliero S J Luhby A L and Yuceoglu P Influence of transfusions on erythropoietic stimulating factor *(ESF)* of anemic patients *Acta haemat* *18* 325-336 1957

220 Van Dyke D C Garcia J F and Lawrence J H Concentration of highly potent erythropoietic activity from urine of anemic patients *Proc Soc Exper Biol & Med* *96* 541 544 1957

221 Winkert J Gordon A S Medici P T Piliero S J Luhby A L and Tannenbaum M Erythropoietic stimulating activity of urine from anemic human subjects *Proc Soc Exper Biol & Med* *97* 191 193 1958

222 Grant W C The influence of anoxia of lactating rats and mice on blood of their normal offspring *Blood* *10* 334 340 1955

223 Stickney J C Browne T L and VanLiere E J Erythropoietic factor in milk of altitude goats *Physiologist* *1* 76 1958

224 Kinard F W and Griffin F F Jr Erythropoietic activity in plasma of anemic dogs *Federation Proc* *17* 86 1958

225 Stohlman F Jr Observations on the physiology of erythropoietin and its role in the regulation of red cell production *Ann New York Acad Sc* *77* 710 724 1959

226 Bonsdorff E On the presence of erythropoietins in the plasma from sheep foetuses during the latter half of gestation *Acta physiol scandinav* *18* 51 62 1949

227 Seip M Humoral factors in regulation of reticulocyte level and erythropoiesis in man *Acta paediat* *44* 507 512 1955

228 Althoff H and Werner H Zum nachweis der erythropoetine beim menschen *Klin Wchnschr* *34* 1197 1198 1956

201 Hodgson G and Toha J The erythropoietic effect of urine and plasma of repeatedly bled rabbits *Blood* 9 299-309 1954

202 Klingelhoffer K O Über die wirkung des sauerstoffs auf das hamopoietin des nabelschnurblutes *Arch ges Physiol* 250 465 473 1948

203 Doring G K and Loeschcke H H Die thermostabilitat des oxydablen hamopoietins aus menschlichen nabel schnurblut *Arch ges Physiol* 251 220 224 1949

204 Loeschcke H H Über die humorale steuerung der normalen erythrozytenbildung *Zschr Vitamin Hormon Fermantforsch* 3 346 359 1950

205 Klingelhoffer K O Über die stabilitat des oxydablen haemopoietins des nabelschnurblutes *Arch ges Physiol* 252 278 280 1950

206 Gunther B Hodgson G Toha J and Quappe O The inactivation by oxygen of the erythropoietic effect of plasma of rabbits rendered anemic by bleeding *Acta Physiol Lat America* 1 271 276 1951

207 Appels A and Keller H M Tierexperimenteller nachweis einer erythrocyten und reticulocyten vermehrenden substanz bei der polycythemia vera *Folia haemat* 1 309-319 1957

208 Erslev A J Observations on the nature of the erythropoietic serum factor II Erythropoietic activity of serum and bone marrow after time limited exposure to anemic and hypoxic anoxia *J Lab & Clin Med* 50 543 549 1957

209 Gley P Nouvelles recherches sur l hematopoietine *Bull Acad nat med* 138 435 440 1954

210 Gley P and Delor J Sur quelques proprietes physicochimiques de l hemapoietine *Compt rend Soc biol* 149 635 637 1955

211 Erslev A J and Lavietes P H Observations on the nature of the erythropoietic serum factor *Blood* 9 1055 1061 1954

212 Gordon A S Influence of humoral factors on erythropoiesis *Am J Clin Nutrition* 5 461 472 1957

213 Slaunwhite W R Jr Mirand E A and Prentice T C Probable polypeptide nature of erythropoietin *Proc Soc Exper Biol & Med* 96 616-619 1957

214 Grant W C Linkenheimer W H and Berger H Concentration of the erythropoietic hormone in plasma of anemic rabbits *Federation Proc* 17 59 1958

243 Lajtha L G Bone marrow cell metabolism *Physiol Rev* *37* 50 65 1957

244 Patt M M A consideration of the myeloid erythroid balance in man *Blood* *12* 777 787 1957

245 Cronkite E P Fliedner T M Bond V P Rubini J R Brecher G and Quastler H Dynamics of hemopoietic proliferation in man and mice studied by H^3 thymidine incorporation into DNA *Ann New York Acad Sc* *77* 803 820 1959

246 Borsook H Deasy C L Haagen Smit A J Keighley G and Lowy P H Incorporation *in vitro* of labeled amino acids into proteins of rabbit reticulocytes *J Biol Chem* *196* 669 694 1952

247 Borsook H Abrams A and Lowy P H Fructose amino acids in liver Stimuli of amino acid incorporation *in vitro* *J Biol Chem* *215* 111 124 1955

248 Borsook H Hemoglobin synthesis *in vitro* in rabbit reticulocytes Conf on Hemoglobin National Academy of Sciences Publication 557 1958 pp 111 130

249 Alpen E L and Cranmore D Cellular kinetics and iron utilization in bone marrow as observed by Fe^{59} radioautography *Ann New York Acad Sc* *77* 753 765 1959

250 Rambach W A Cooper J A D and Alt H L Effect of hypoxia on DNA synthesis in the bone marrow and spleen of the rat *Science* *119* 380 381 1954

251 Erslev A J The effect of anemic anoxia on the cellular development of nucleated red cells *Blood* *14* 386 398 1959

252 Erslev A J Erythropoietic factor in the control of red cell production *Ann New York Acad Sc* *77* 627 637 1959

253 Osgood E E and Brownlee I E Culture of human marrow Details of a simple method *J A M A* *108* 1793 1796 1937

254 Osgood E E Blood cell survival in tissue cultures *Ann N Y Acad Sc* *77* 777 796 1959

255 Thomas E D and Lochte H L Jr Studies on the biochemical defect of pernicious anemia I *In vitro* observations on oxygen consumption heme synthesis and desoxyribonucleic acid synthesis by pernicious anemia bone marrow *J Clin Investigation* *37* 166 171 1958

256 Bertinchamps A Kenis Y Tagnon H J and Peltzer T The effect of HN on hemoglobin synthesis by avian erythrocytes *in vitro* *Cancer* *11* 996 998 1958

229 Althoff H and Werner H Vorkommen und bedeutung der erythropoietine bei der erythroblastosis foetalis *Acta haemat* *18* 126 136 1957

230 Biber T Über den nachweis von hamopoietin im menschlichen blut bei hohenaufenthalt *Helvet physiol acta* *15* 408-418 1957

231 Althoff H Dahm P and Werner H Über das vorkommen der erythropoetine im nabelschnurblut *Arch Kinderh* *157* 238 244 1958

232 Krantz S Goldwasser E and Jacobson L O Studies on erythropoiesis XIV The relationship of humoral stimulation to iron absorption *Blood* *14* 654 661 1959

233 Evans J D Baker J M and Oppenheimer M J Alterations of rabbit marrow fat in anemia from acetylphenylhydrazine *Am J Physiol* *181* 504 508 1955

234 Evans J D and Oppenheimer M J Effect of bleeding on rabbit marrow polyunsaturated fatty acids *Am J Physiol* *181* 509-512 1955

235 Gley P Double aspect of the hematopoietic hormone of serum *Proc VI Cong Internat Soc Hemat* New York Grune and Stratton 1958 p 785

236 Japa J A study of the mitotic activity of normal human bone marrow *Brit J Exper Path* *23* 272 276 1942

237 Kindred J E A quantitative study of the hemopoietic organs of young adult albino rats *Am J Anat* *71* 207 243 1942

238 Dacie J V and White J C Erythropoiesis with particular reference to its study by biopsy of human bone marrow A review *J Clin Path* *2* 1 32 1949

239 Schwarz E Maturation and proliferation in normo and megaloblastic erythropoiesis *Acta med scandinav* *139* 445 464 1951

240 Widner W R Storer J B and Lushbaugh C C The use of x ray and nitrogen mustard to determine the mitotic and intermitotic times in normal and malignant rat tissues *Cancer Res* *11* 877-884 1951

241 Osgood E E Drug induced hypoplastic anemias and related syndromes *Ann Int Med* *39* 1173 1188 1953

242 Osgood E E Number and distribution of human hemic cells *Blood* *9* 1141 1154 1954

272 Prelog V Ruzicka L and Stein P Zur kenntnis der unverseif baren lipoide aus schweinemilz *Helvet chim acta 26* 2222 2242 1943
273 Hardegger E Ruzicka L and Tagmann E Zur kenntnis der unverseifbaren lipoide aus arteriosklerotischen aorten *Helvet chim acta 26* 2205 2221 1943
274 Bodman J and Maisin J H The α glyceryl ethers *Clinica chimica acta 3* 253 274 1958
275 Linman J W Bethell F H and Long M J The erythropoi etic stimulatory activity of batyl alcohol *J Lab & Clin Med* 52 596 604 1958
276 Evenstein D Gordon A S and Eisler M Quoted in Gordon A S Hemopoietine *Physiol Rev* 39 1-40 1959
277 Miller C O Skoog F Okumura F S Von Saltza M H and Strong F M Isolation structure and synthesis of kinetin a substance promoting cell division *J A C S 78* 1375 1380 1956
278 Tannenbaum M and Gordon A S Influence of erythropoietic stimuli upon enzymatic patterns of bone marrow and plasma *Proc VI Cong Internat Soc Hemat* New York Grune and Stratton 1958 pp 783 784
279 Steinberg B Bone marrow regeneration in experimental ben zene intoxication *Blood 4* 550 556 1949
280 Deuel H J Jr *The Lipids Their Chemistry and Biochemistry* Vol I New York Interscience Publishers Inc 1951 pp 392 396
281 Edlund T Protective effect of d l α octadecylglycerolether in mice given total body irradiation *Nature 174* 1102 1954
282 Brohult A and Holmberg J Alkoxyglycerols in the treatment of leukopenia caused by irradiation *Nature 174* 1102 1103 1954
283 Brohult A Alkoxyglycerol esters in irradiation treatment *Ad vances in Radiobiology* Stockholm Conf Edinburgh Oliver and Boyd Ltd 1957 pp 241 247
284 Evans W C Evans I A Edwards C M and Thomas A J Bracken poisoning of cattle—therapeutic treatment *Biochem J 65* 5 1957
285 Schultze M O Perman V Bates F W and Sautter J H Failure of dl batyl alcohol to prevent aplastic anemia in calves *Proc Soc Exper Biol & Med* 98 470 474 1958

257 Swann M M The control of cell divisions A review I General mechanisms *Cancer Res* 17 727 757 1957
258 Swann M M The control of cell division A review II Special mechanisms *Cancer Res* 18 1118 1160 1958
259 Steele B F The effects of blood loss and blood destruction upon the erythroid cells in the bone marrow of rabbits *J Exper Med* 57 881 896 1933
260 Huff R L Lawrence J H Siri W E Wasserman L R and Hennessy T G Effect of changes in altitude on hematopoietic activity *Medicine* 30 197 217 1951
261 Stohlman F Jr The utilization of erythropoietin *Clin Res Proc* 6 193 194 1958
262 Stohlman F Jr and Brecher G Humoral regulation of erythropoiesis V Relationship of plasma erythropoietine level to bone marrow activity *Proc Soc Exper Biol & Med* 100 40-43 1959
263 Singer K The lysolecithin fragility test *Am J M Sc* 199 466 477 1940
264 Bethell F H and Blecl K The production of microspherocytosis of red cells and hemolytic anemia by the injection of rattlesnake (Crotalus atrox) venom *J Clin Investigation* 21 641 1942
265 Wilbur K M and Collier H B A comparison of the hemolytic actions of lysolecithin and saponin *J Cell & Comp Physiol* 22 233 249 1943
266 Tompkins E H Effects of repeated intravenous injections of lecithin in rabbits The relationships to lipoid storage diseases and to hemolytic anemias *Arch Path* 35 695 712 1943
267 Ponder E *Hemolysis and Related Phenomena* New York Grune and Stratton 1948
268 Rouse P Destruction of the red blood corpuscles in health and disease *Physiol Rev* 3 75 105 1923
269 Thomas E D *In vitro* studies of erythropoiesis I The effect of normal serum on heme synthesis and oxygen consumption by bone marrow *Blood* 10 600 611 1955
270 Ponder E The rate of development of red cell precursors *Science* 102 257 258 1945
271 Holmes H N Corbet R E Geiger W B Kornblum N and Alexander W The isolation and identification of batyl alcohol and cholesterol from yellow bone marrow *J A C S* 63 2607 2609 1941

300 Forster J and Kiss F Untersuchungen uber die fordernde wirkung des anamischen blutes auf die blutkorperchenbildung *Biochem Ztschr* *160* 442 447 1925

301 Davis J E McCullough A W and Rigdon R H Polycythemia produced by cobalt in the duck *J Lab & Clin Med* *30* 327-336 1945

302 Levy H Levison V and Schade A L The effect of cobalt on the activity of certain enzymes in homogenates of rat tissue *Arch Biochem* *27* 34-40 1950

303 Laforet M T and Thomas E D The effect of cobalt on heme synthesis by bone marrow *in vitro* *J Biol Chem* *218* 595 598 1956

304 Goldwasser E Jacobson L O Fried W and Plzak L Mechanism of the erythropoietic effect of cobalt *Science* *125* 1085 1086 1957

305 Goldwasser E Jacobson L O Fried W and Plzak L F Studies on erythropoiesis V The effect of cobalt on the production of erythropoietin *Blood* *13* 55 60 1958

306 Brown T E and Meineke H A Presence of an active erythropoietic factor (erythropoietin) in plasma of rats after prolonged cobalt therapy *Proc Soc Exper Biol & Med* *99* 435 437 1958

307 Erslev A J Erythropoietic function in dilution anemia *Blood* *10* 616 622 1955

308 Contopoulos A N Van Dyke D C and Simpson M E Increased erythropoietic stimulant in plasma of pregnant rats *Proc Soc Exper Biol & Med* *93* 424 428 1956

309 Loeschcke E and Schwartzer K Untersuchungen zur frage der fetalen polyglobulie *Monatsschr Kinderh* *81* 25 28 1939

310 Stohlman F Jr Sarnoff S J Case R B and Ness A T Hemolytic syndrome following the insertion of a lucite ball valve prosthesis into the cardiovascular system *Circulation* *13* 586 591 1956

311 Stohlman F Jr and Sarnoff S J Comparison of hemolysis following the insertion of lucite and silastic ball valves into the circulation system *Proc VI Cong Internat Soc Hemat* New York Grune and Stratton 1958 pp 807 812

312 Brecher G and Stohlman F Jr Humoral factors in erythropoiesis *Prog in Hematology* Vol 2 New York Grune and Stratton 1959 pp 110 132

286 Giffin H Z and Watkins C H The administration of yellow bone marrow in agranulocytic angina *Minnesota Med*, *21* 62 63 1938

287 Marberg C M and Wiles H O Granulocytopoietic fraction of yellow bone marrow *Arch Int Med 61* 408-429 1938

288 Caldwell J E Sifferd R H Porsche J D and Fenger F Recent studies on yellow bone marrow extracts *Am J M Sc* *209* 717 721 1945

289 Gordon A S Piliero S J Tannenbaum M and Siegel C D Effects of an erythropoietic plasma filtrate upon blood formation in the hypophysectomized rat *Anat Rec 122* 491-492 1955

290 Nakao H Fortgestzte untersuchungen uber die beziehungen zwischen schildruse thymus milz und knochenmark *Biochem Ztschr 166* 337 349 1925

291 Krumdieck N Erythropoietic substance in serum of anemic animals *Proc Soc Exper Biol & Med 54* 14 17 1943

292 Kinard F W and Ellis D W Reticulocytosis following injection of anoxic plasma in dogs *Anat Rec 105* 556 1949

293 Gray D F and Ersley A J Reticulocytosis induced by serum from hypoxic animals *Proc Soc Exper Biol & Med 94* 283 286 1957

294 Stohlman F Jr and Brecher G Humoral regulation of erythropoiesis III Effect of exposure to simulated altitude *J Lab & Clin Med 49* 890 895 1957

295 Mirand E A and Prentice T C Presence of plasma erythropoietin in hypoxic rats with and without kidney(s) and/or spleen *Proc Soc Exper Biol & Med 96* 49 51 1957

296 Stohlman F Jr Rath C E and Rose J C Evidence for a humoral regulation of erythropoiesis Studies on a patient with polycythemia secondary to regional hypoxia *Blood 9* 721 733 1954

297 Schmid R and Gilbertsen A S Fundamental observations on the production of compensatory polycythemia in a case of patent ductus arteriosus with reversed blood flow *Blood 10* 247 251 1955

298 Sugerman D A The bone marrow in ischaemia *M J Australia 2* 913 917 1956

299 Reynafarje C The influence of high altitude on erythropoietic activity Brookhaven Symposia in Biology No 10 Homeostatic Mechanisms *1958 pp 132 146*

326 Naets J P The kidney and erythropoiesis *Nature 182* 1516-1517 1958
327 Naets J P Erythropoiesis in nephrectomized dog *Experientia 14* 74 1958
328 Richet G Alagille D and Fournier E L erythroblastopenie aigue de l anurie *Presse med* 62 50 53 1954
329 Erslev A J Erythropoietic function in uremic rabbits *Arch Int Med 101* 407-417 1958
330 Muirhead E E Jones F and Grollman A The anemia of renal insufficiency as induced by bilateral nephrectomy of the rabbit With emphasis on its hemolytic nature *J Lab & Clin Med* 39 505 517 1952
331 Callen I R and Limarzi L R Blood and bone marrow studies in renal disease *Am J Clin Path* 20 3 23 1950
332 Loge J P Lange R D and Moore C V Characterization of the anemia associated with chronic renal insufficiency *Am J Med 24* 4 18 1958
333 Desforges J F and Dawson J P The anemia of renal failure *Arch Int Med 101* 326-332 1958
334 Cooper W M and Tuttle W B Polycythemia associated with a benign kidney lesion Report of a case of erythrocytosis with hydronephrosis with remission of polycythemia following nephrectomy *Ann Int Med 47* 1008 1015 1957
335 Forssell J Nephrogenous polycythemia *Acta med scandinav 161* 169 179 1958
336 Lawrence J H and Donald W G Jr Polycythemia and hydronephrosis or renal tumors *Ann Int Med* 50 956 969 1959
337 Van Lessen H G Stefanini M and Smith F E Erythrocytosis after unilateral partial ligation of renal vein in dogs *Clin Res Proc 6* 193 1958
338 Lange R D Gallagher N I McCarthy J M and Wright S Myelotoxicity nephrotoxicity and erythropoietic stimulating factor production in animals treated with aminonucleoside of puromycin *J Lab & Clin Med* 53 912 920 1959
339 Gallagher N I McCarthy J M Hart K T and Lange R D Evaluation of plasma erythropoietic stimulating factors in anemic uremic patients *Blood 14* 662 667 1959
340 Reynafarje C Lozano R and Valdivieso J The polycythemia of high altitudes Iron metabolism and related aspects *Blood 14* 433 455 1959

313 Kleinberg W Gordon A S and Charipper H A Effect of cobalt on erythropoiesis in anemic rabbits *Proc Soc Exper Biol & Med* 42 119 120 1939

314 Cohn E W and D Amour F E Effects of high altitude and cobalt on growth and polycythemia in rats *Am J Physiol* 166 394-399 1951

315 Altland P D and Parker M Effects of hypoxia upon the box turtle *Am J Physiol* 180 421 427 1955

316 Tannenbaum M and Gordon A S Influence of hemorrhage on peptidase activity of blood and bone marrow *Federation Proc* 15 184 185 1956

317 Prentice T C and Mirand E A Effect of acute liver damage plus hypoxia on plasma erythropoietin content *Proc Soc Exper Biol & Med* 95 231 234 1957

318 Jacobson L O Goldwasser E Gurney C W Fried W and Plzak L Studies on erythropoietin The hormone regulating red cell production *Ann New York Acad Sc* 77 551 573 1959

319 Bonsdorff E and Seleste F Artificially induced local anoxaemia in the treatment of childrens anaemia *Acta paediat* 37 441 448 1949

320 Hippo A Treatment of anaemia with plasma from deoxygenated blood *Acta paediat* (Supp 77) pp 169 173 1949

321 Mathe G and Bernard J Effet du facteur erythropoietique libere par la saignée sur la correction de l anemie provoquee par l irradiation X *Rev hemat* 12 507 517 1957

322 Crafts R C and Meineke H A Presence of erythropoietic factor in plasma of normal and hypophysectomized rats following bleeding *Proc Soc Exper Biol & Med* 29 222 224 1956

323 Goldwasser E Fried W and Jacobson L O Studies on erythropoiesis VIII The effect of nephrectomy on response to hypoxic anoxia *J Lab & Clin Med* 52 375-378 1958

324 Jacobson L O Marks E K Gaston E O and Goldwasser E Studies on erythropoiesis XI Reticulocyte response of transfusion induced polycythemic mice to anemic plasma from nephrectomized mice and to plasma from nephrectomized rats exposed to low oxygen *Blood,* 14 635 643 1959

325 Naets J P Erythropoiesis in nephrectomized dogs *Nature* 181 1134 1135 1958

354 Schulhof K and Matthies M M Polyglobulia induced by cerebral leisons *J A M A* *89* 2093 2094 1927

355 Moehlig R C and Bates G S Influence of the pituitary gland on erythrocyte formation *Arch Int Med* *51* 207 235 1933

356 Carpenter G Schwartz H G and Walker A E Neurogenic polycythemia *Ann Int Med* *19* 470-481 1943

357 Drew J H and Grant F C Polycythemia as neurosurgical problem review with reports of 2 cases *Arch Neurol & Psychiat* *54* 25-36 1945

358 Haynal E and Graf F The role of the hypophyseal hypo thalamic system in the pathogenesis of erythraemia and symp tomatic polycythaemias *Acta med scandinav* *139* 61 77 1950

359 Blumberg B and Myerson R M Erythrocytosis associated with cerebellar hemangioblastoma *Neurology* *7* 367-369 1957

360 Linman J W and Bethell F H Observations on the role of the plasma erythropoietic factors in polycvthemia vera *J Lab & Clin Med* *50* 927 928 1957

361 Linman J W Korst D R and Bethell F H Some observa tions on the stimulation of erythropoiesis by humoral factors *Ann New York Acad Sc* *77* 638 649 1959

362 Berlin N I Lawrence J H and Lee H C The life span of the red blood cell in chronic leukemia and polycythemia *Science* *114* 385-387 1951

363 Huff R L Hennessy T C Austin R E Garcia J F Roberts B M and Lawrence J H Plasma and red cell iron turn over in normal subjects and in patients having various hemato poietic disorders *J Clin Investigation* *29* 1041 1052 1950

364 Wasserman L R Rashkoff I A Leavitt D Mayer J and Port S The rate of removal of radioactive iron from the plasma—an index of erythropoiesis *J Clin Investigation* *31* 32 39 1952

365 Minot G R and Buckman T E Erythremia (polycythemia rubra vera) The development of anemia the relation to leukemia consideration of the basal metabolism blood forma tion and the destruction and fragility of the red cells *Am J M Sc* *166* 469-489 1923

341 Vaquez M H Sur une forme speciale de cyanose s accompagnant d hyperglobulie excessive et persistante *Compt rend Soc biol 44* 384 388 1892

342 Saundby R and Russell J W An unexplained condition of chronic cyanosis with report of a case *Lancet 1* 515 517 1902

343 Osler W Chronic cyanosis with polycythemia and enlarged spleen A new clinical entity *Am J M Sc 126* 187 201 1903

344 Lawrence J H Berlin N I and Huff R L The nature and treatment of polycythemia Studies on 263 patients *Medicine 32* 323-388 1953

345 Wasserman L R Polycythemia vera—its course and treatment Relation to myeloid metaplasia and leukemia *Bull New York Acad Med 30* 343 375 1954

346 Lawrence J H *Polycythemia Physiology Diagnosis and Treatment Based on 303 Cases* Modern Medical Monograph New York Grune and Stratton 1955

347 Calabresi P and Meyer O O Polycythemia vera I Clinical and laboratory manifestations *Ann Int Med 50* 1182 1202 1959

348 Calabresi P and Meyer O O Polycythemia vera II Course and therapy *Ann Int Med 50* 1203 1216 1959

349 Berk L Burchenal J H Wood T and Castle W B Oxygen saturation of sternal marrow blood with special reference to pathogenesis of polycythemia vera *Proc Soc Exper Biol & Med 69* 316 320 1948

350 Schwartz B M and Stats D Oxygen saturation of sternal marrow blood in polycythemia vera *J Clin Investigation 28* 736 740 1949

351 Robertson J S Siri W E and Jones H B Lung ventilation patterns determined by analysis of nitrogen elimination rates use of the mass spectrometer as a continuous gas analyzer *J Clin Investigation 29* 577 590 1950

352 Lawrence J H Elmlinger P J and Fulton G Oxygen and control of red cell production in primary and secondary polycythemia Effects on the iron turnover patterns with Fe^{59} as tracer *Cardiologia 21* 337 346 1952

353 Reznikoff P Foot N C and Bethea J M Etiologic and pathologic factors in polycythemia vera *Am J M Sc 189* 753 759 1935

INDEX

366 Sturgis C C *Hematology* 2nd ed Springfield Thomas 1955 p 1059
367 Isaacs R Pathologic physiology of polycythemia vera *Arch Int Med* *31* 289 296 1923
368 Daland G A and Worthley K The resistance of red blood cells to hemolysis in hypotonic solutions of sodium chloride Observations in blood disorders *J Lab & Clin Med* *20* 1122 1136 1935
369 Linman J W Bethell F H and Long M J Factors controlling hemopoiesis Experimental observations on their role in polycythemia vera *Ann Int Med* *51* 1003 1018 1959
370 Schulman I Pierce M Lukens A Currimbhoy Z and Fort, E A factor in normal human plasma required for platelet production Chronic thrombocytopenia due to its deficiency *Proc Soc Ped Res* Buckhill Falls Pa May 8 9 1959, p 64

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